Published in final edited form as:

Mol Carcinog. 2007 May ; 46(5): 341–353. doi:10.1002/mc.20282.

Colorectal Carcinoma Development in Inducible Nitric Oxide Synthase-Deficient Mice With Dextran Sulfate Sodium-Induced Ulcerative Colitis

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Abstract

The overproduction of reactive oxygen and nitrogen species (RONS) may play an important role in ulcerative colitis (UC)-associated carcinogenesis. In order to study the role of nitric oxide (NO) in UC-associated colorectal carcinogenesis, the development of colorectal carcinoma was studied using the DSS-induced and iron-enhanced model of chronic UC in inducible nitric oxide synthase (*iNOS*)-deficient mice. Female wild-type C57BL/6 (*iNOS*^{+/+}) and *iNOS*^{-/-} mice were administered 1% DSS (w/v) through the drinking fluid for 15 DSS cycles and fed twofold iron-enriched diet. Colorectal inflammation and mucosal ulceration of moderate severity were observed in both *iNOS*^{+/+} and *iNOS*^{-/-} mice. Similar tumor incidence and multiplicity in the colon were observed that 15 out of 23 (65.2%) *iNOS*^{+/+} mice developed colorectal tumors with a tumor multiplicity of 1.47 ± 0.17 (mean $\pm$ SE) after 15 DSS cycles, and 13 out of 19 (68.4%) *iNOS*^{-/-} mice developed colorectal tumors with a tumor multiplicity of 2.08 ± 0.21 . Histopathologically, the tumors were confirmed to be well-differentiated adenocarcinomas. Nitrotyrosine, an indicator of peroxynitrite-caused protein modification, was detectable by immunohistochemistry in inflammatory cells and epithelial cells of the colon in *iNOS*^{+/+} and *iNOS*^{-/-} mice, and no difference in staining intensity was observed between the two groups. Immunostaining for endothelial NOS (eNOS) was observed in lamina propria macrophages and colonic blood vessels, and eNOS protein levels were increased in the inflamed colon. These results show that there is no difference in UC-associated cancer development in *iNOS*^{+/+} and *iNOS*^{-/-} mice, and suggest that in the absence of iNOS, other factors, such as eNOS, may play a role in nitrosative stress and UC-associated carcinogenesis in this model system.

Keywords

ulcerative colitis; carcinogenesis; iNOS; eNOS; nitrotyrosine

INTRODUCTION

Long-standing ulcerative colitis (UC) is known to confer an increased risk for colorectal cancer, presumably as a result of long-standing inflammation [1–4]. Chronic UC is characterized by colonic nitrosative and oxidative stress because of the overproduction of reactive oxygen and nitrogen species (RONS) and a depletion of antioxidant molecules [5–10]. RONS may contribute to the initiation and promotion of carcinogenesis in the setting of UC through mutagenic and pro-mitogenic activities [11,12].

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The enzyme nitric oxide synthase (NOS) catalyzes the formation of nitric oxide (NO) and L-citrulline by the reduction of L-arginine [13]. The inducible isoform of NOS (iNOS), originally identified in macrophages, exhibits calcium-independent function and is so named because iNOS gene expression is induced by pro-inflammatory cytokines. The activation of iNOS leads to prolonged production of NO in high, potentially cytotoxic concentrations [14]. Inducible NOS serves a defensive function in the host inflammatory response to microorganisms [15]. However, excessive production of NO under inflammatory conditions may be detrimental by mediating damage to nearby native cells. It has been shown that iNOS is overexpressed in the colons of UC patients, and may very well contribute to the pathogenesis of UC [8,16–19].

NO may also play a role in the enhancement of colon cancer risk in UC patients [11,20,21]. NO and peroxynitrite, the product of the reaction of NO with the superoxide anion, are mutagenic in vitro, taking part in DNA nitrosation and subsequent deamination reactions, as well as oxidative reactions. NO may also indirectly facilitate carcinogenesis by inhibiting the functions of thiol-containing DNA repair proteins [22], and by stimulating new blood vessel formation via increases in vascular permeability and endothelial cell proliferation [11,23]. The spectrum of mutations in the p53 tumor suppressor gene in UC-associated colorectal carcinomas and dysplasias is consistent with RONS-mediated DNA alterations [24,25]. However, evidence directly demonstrating roles for iNOS and NO in UC-associated carcinogenesis has been lacking.

The oral administration of dextran sulfate sodium (DSS) to rodents induces colonic inflammation that is clinically and histologically similar to human UC [26]. Like the human disease, DSS-induced chronic UC is complicated by the development of colorectal dysplasia and adenocarcinoma [27–30]. We have shown that dietary iron supplementation enhances colorectal carcinoma development in the DSS model, an effect that is associated with increases in nitrotyrosine formation and iNOS expression by colonic inflammatory cells [30]. In addition, the antioxidant *N*-acetylcysteine had an inhibitory effect on nitrotyrosine formation and colorectal carcinogenesis in mice with DSS-induced and iron-enhanced UC [31]. These results suggest that NO participates in inflammation-caused cellular injury in this model system.

In order to study the mechanistic roles of iNOS and NO in UC-associated carcinogenesis, tumor development, inflammation, and protein nitrosation were analyzed in *iNOS*-deficient mice with DSS-induced, iron-enhanced chronic UC. The possible involvement of endothelial NOS (eNOS) in inflammation-caused protein nitrosation in the *iNOS* knockout mouse was also studied using immunohistochemical and Western blot approaches.

MATERIALS AND METHODS

Animals

Six-week-old female B6/129P-Nos2^{tm1Lau} (*iNOS*^{−/−}, C57BL/6 background) [32] and wild-type C57BL/6 (*iNOS*^{+/+}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The genotypes were confirmed by polymerase chain reaction of tail DNA using specific oligonucleotide primers (primer sequences obtained from the Jackson Laboratories Mouse Genome Informatics web-site: www.informatics.jax.org). The mice were housed five per cage under pathogen-free conditions in the facilities of Laboratory Animal Services, Rutgers University. All mice were acclimated to the animal room conditions for 2 wk prior to the start of the experiment, during which time they were administered ordinary lab chow and tap water ad libitum. Body weights and food consumption were monitored every other week for the duration of the experiment.

Chemicals and Diet

DSS (MW: ~ 40 000) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Mayer's hematoxylin solution, eosin Y solution, and 3, 3'-diaminobenzidine (DAB) were from Sigma Chemical Co. (St. Louis, MO). Antigen Unmasking Solution (AUS) and avidin/biotinylated horseradish peroxidase macromolecular complex kits were from Vector Laboratories (Burlingame, CA). Polyclonal anti-CD45 and anti-Mac3 were from BD Pharmingen (San Diego, CA). Polyclonal anti-iNOS (anti-NOS2, antibody M-19), and polyclonal anti-eNOS (anti-NOS3, antibody C-20) were from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA). Polyclonal anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-proliferating cell nuclear antigen (PCNA) was from Oncogene Science, Inc. (San Diego, CA). Polyclonal anti-von Willebrand factor (vWF) was from Dako, Inc. (Carpinteria, CA). AIN76A-based diet containing 45 mg extra iron per kg diet (total: 90 mg iron per kg diet) was purchased from Research Diets, Inc. (New Brunswick, NJ).

Animal Experiments

In a long-term study of chronic UC and associated colorectal carcinogenesis, *iNOS*^{+/+} and *iNOS*^{-/-} mice were randomized into four groups. Group 1 (*iNOS*^{+/+}) and Group 2 (*iNOS*^{-/-}) were administered water throughout the experiment as negative controls (*n* = 5 mice per group). Group 3 (*iNOS*^{+/+}) and Group 4 (*iNOS*^{-/-}) mice (*n* = 25 mice per group) were subjected to cyclic DSS treatment [26,30,31]. One DSS "cycle" consisted of 7 days of 1% DSS (w/v in deionized water) administration through the drinking fluid (DSS period), followed by 10 days of ordinary tap water administration (recovery period). The negative control groups were given deionized water during the DSS period and ordinary tap water during the recovery period. The mice were subjected to 15 consecutive DSS cycles. Starting 2 days before the start of the experiment and continuing for the duration of the experiment, all groups were given a modified AIN76A diet containing 90 mg iron per kg diet (AIN76A/2XFe diet; purchased from Research Diets, Inc.). The mice were sacrificed at the end of the 15th DSS cycle (Day 255). Mice were sacrificed prior to the end of the experiment period if they exhibited significant weight loss (greater than 20%), excessive rectal bleeding, and loss of activity. Mice sacrificed prior to the completion of 15 DSS cycles were excluded from the results of the carcinogenesis experiment.

In a short-term study, 10 *iNOS*^{+/+} and 10 *iNOS*^{-/-} mice were divided into four groups. Group 1 (*iNOS*^{+/+}) and Group 2 (*iNOS*^{-/-}) were administered water as the negative control (*n* = 5 mice per group). Group 3 (*iNOS*^{+/+}) and Group 4 (*iNOS*^{+/+}) mice (*n* = 5 mice per group) were given 1% DSS in the drinking fluid for 1 cycle (7 days DSS followed by 10 days of tap water consumption). The negative control mice were administered distilled water during the DSS period and tap water during the recovery period. All groups were fed AIN76A/2XFe diet starting 2 days before the start of the experiment and continuing until the end of the experiment. The mice were sacrificed on Day 17. The colon from each mouse was removed and divided in half longitudinally. One half of the colon was fixed in formalin for histopathological analysis, and the remaining half was frozen and stored at -80°C for use in Western blot analysis.

Tissue Preparation and Histopathological Evaluation

After sacrificing the mice by CO₂ asphyxiation, the colons were perfused with 10% neutral buffered formalin, removed, and opened longitudinally. The numbers and positions of colon tumors were recorded, and three perpendicular diameter measurements were obtained for each tumor using calipers. The tumor volume was determined using the equation $V = 4/3\pi r^3$, where *r* was the average tumor radius obtained from the three diameter measurements. The colons were then fixed in 10% formalin for 24 h, routinely processed, and embedded in paraffin as a "Swiss roll." Serial tissue sections (5 μm) were made and stained with hematoxylin and eosin (H&E) for histopathological analysis, or used for immunohistochemical staining.

Colorectal tumors were analyzed by light microscopy and classified as colorectal adenocarcinoma according to established criteria [30]. The tumors were further categorized as tubular well-differentiated adenocarcinoma or mucinous well-differentiated adenocarcinoma based on the glandular architecture and presence or absence of mucin lakes.

Histological Grading of UC

H&E-stained serial tissue slides were used for histological scoring of the UC index in the proximal, middle, and distal thirds of the colon. As compared to the colons of negative control mice (morphologically normal mucosa, Figure 1A), the colons of mice treated with DSS/2Xfe showed both non-actively-inflamed mucosa (showing mild hyperplastic change with glandular distortion and a few inflammatory cell infiltrates; Figure 1B) and actively-inflamed mucosa (showing erosion, ulceration, epithelial injury, and epithelial regeneration with abundant inflammatory cell infiltrates; Figure 1C and D). The UC index was obtained from the scores for inflammation severity, ulceration, hyperplasia, and area of inflammatory involvement. The criteria for the scoring of these parameters have been described previously [30,33]. The mean score for each parameter was obtained from the individual scores for five to six mice per group. The total score (UC index) was the sum of the individual parameter scores for each mouse. The mean UC index for each group was the average of the total scores for five to six mice per group.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin–biotin–peroxidase complex method as described previously [31]. In brief, endogenous peroxidase activity was quenched in paraffin-embedded tissue sections with 1% H₂O₂. Antigen was retrieved by pretreatment with citrate buffer (Antigen Unmasking Solution, Vector Laboratories, Burlingame, CA) in a microwave oven. Non-specific protein–protein interactions were blocked with diluted normal serum, then the slides were incubated with primary antibody (5 µg/mL for anti-CD45 and anti-Mac-3, 1:1000 dilution for polyclonal anti-nitrotyrosine, 2 µg/mL for polyclonal anti-eNOS and monoclonal anti-PCNA, 1:200 dilution for anti-vWF), followed by the appropriate biotinylated secondary antibody and the ABC complex for 45 min each. Diaminobenzidine was used as the chromagen. Slides were washed thoroughly between incubations with PBS. Negative controls were established by replacing the primary antibody with PBS and normal serum. Positive staining was indicated by the presence of brown-colored precipitate.

Assessment of Inflammatory Cell Infiltration by CD45 and Mac3-Staining

The mucosal infiltration of inflammatory cells was assessed by immunohistochemical staining for CD45 (majority of positive cells are lymphocytes) and Mac3 (macrophages). The densities of CD45 and Mac3-positive cells in the colonic mucosa were defined as the number of immunostain-positive cells per mm² of mucosa. Positive cell numbers, and mucosal and tumor areas, were measured using a Nikon research microscope in conjunction with a Nikon DXM 1200 digital camera and image analysis software (Image-Pro Plus Version 4.5, Media Cybernetics, Silver Spring, MD). The immunostain-positive cell density was determined in six microscope fields (20× objective lens) of the distal colon or tumor per mouse. Slides from five randomly chosen mice per group were analyzed.

PCNA Labeling Index

Immunohistochemistry for PCNA was used as a measure of cellular proliferation. The PCNA labeling index was determined for the epithelia in non-actively inflamed mucosa and actively inflamed mucosa in the distal colon, and for tumors. The proliferation index was defined as the ratio of the number of PCNA immunostain-positive cells and the total number of epithelial

or tumor cells counted. Slides from six mice per group were analyzed and greater than 1000 epithelial cells or tumor cells were counted per slide.

von Willebrand Factor (vWF)-Positive Blood Vessel Density

Blood vessel density was assessed in non-actively inflamed or actively inflamed mucosa in the distal colon, or within colonic tumors, by immunostaining for vWF. The vessel density was defined as the number of vWF immunostain-positive blood vessels per area of mucosa or tumor. Mucosal and tumor areas were measured using image analysis software. The vessel density was determined in six microscope fields ($20 \times$ objective) of distal colon mucosa or tumor per mouse. Slides from five randomly chosen mice per group were analyzed.

Western Blot Analysis

Colon samples were minced with scissors, homogenized in ice-cold homogenization buffer, and sonicated. Tissue homogenates were centrifuged at 1000g for 15 min and the tissue debris was discarded. Supernatants were further heated at 95°C in sample buffer, separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. The blots were checked for equal protein loading by staining the membranes with Ponceau S solution. The membranes were incubated in 5% non-fat milk in TBS/0.1% Triton X-100 for 2 h at room temperature to minimize non-specific protein-protein interactions, and washed between incubations with TBS/0.1% Triton X-100. Inducible NOS and eNOS were detected by overnight incubation in primary antibody (1:500 dilution for anti-iNOS and anti-eNOS; 1:5000 dilution for anti-actin) diluted in TBS/0.5% non-fat milk/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

The data were expressed as the mean $\pm$ SE. Statistical significance was tested using SigmaStat Version 1.01 software (Jandel Scientific, San Rafael, CA). Tumor incidence was analyzed using Chi-square test. Normally distributed data were analyzed using Student's *t*-test, and non-parametric data, such as tumor multiplicity, were analyzed using the Mann-Whitney Rank Sum test. *P* values less than 0.05 were taken to be statistically significant.

RESULTS

Body Weight, Food Consumption, and Mortality

In a long-term animal experiment, the average body weight of Group 2 *iNOS*^{-/-} control mice did not differ from that of their Group 1 *iNOS*^{+/+} counterparts at the end of the experiment (mean $\pm$ SE, 22.1 $\pm$ 0.4 vs. 22.9 $\pm$ 0.5 g, respectively). The average body weights for Groups 3 and 4 at the end of the experiment (22.8 $\pm$ 0.5 and 22.1 $\pm$ 0.4 g, respectively) did not differ from each other or from the negative control groups. Similarly, there were no differences in average food consumption among the four groups (data not shown). Two mice from Group 3 and six mice from Group 4 were sacrificed or died prior to the end of the experiment because of severe diarrhea and rectal bleeding and were excluded from the results of the carcinogenesis experiment. Histological examination of the colons from these mice showed extensive ulceration.

UC-Associated Colorectal Cancer Development in *iNOS*^{+/+} and *iNOS*^{-/-} Mice

Groups 1 and 2 negative control mice did not exhibit colorectal tumors. The colorectal tumorigenesis results for Groups 3 and 4 are shown in Table 1. Fifteen out of 23 (65.2%) Group 3 *iNOS*^{+/+} mice administered DSS and twofold iron diet (DSS/2XFe) harbored colorectal

tumors. The average number of colorectal tumors per tumor-bearing mouse (tumor multiplicity) for Group 3 was 1.47 ± 0.17 (mean $\pm$ SE). The average tumor volume for this group was 0.03 ± 0.01 cm³, with a range of 0.001 cm³ to 0.16 cm³. Thirteen out of 19 (68.4%) *iNOS*^{-/-} mice in Group 4 had colorectal tumors. The tumor multiplicity for Group 4 was 2.08 ± 0.21 tumors per tumor-bearing mouse and the average tumor volume was 0.031 ± 0.01 cm³ (range: 0.002 cm³ to 0.25 cm³) (Table 1). Statistical analysis showed there was no difference in tumor incidence, multiplicity, or volume between Groups 3 and 4.

The colorectal tumors were examined in serial H&E-stained slides by light microscopy and classified as well-differentiated adenocarcinomas. Most of the tumors were invasive into the muscle layer, and frequently into the serosa. Nineteen out of 22 (86.4%) tumors in Group 3, and 26 out of 27 (96.3%) in Group 4, were categorized as mucinous well-differentiated adenocarcinoma (Figure 1E and F). Three out of 22 (13.6%) and 1 out of 27 (3.7%) tumors in Groups 3 and 4, respectively, were polypoid well-differentiated tubular adenocarcinomas (Figure 1G and H). Of the 22 tumors in Group 3 mice, 7 were located in the middle third of the colon and 15 were in the distal third. Rectal adenocarcinomas accounted for 10 of the distal tumors in Group 3. Thirteen of the 27 tumors in Group 4 mice were located in the middle colon, while 14 were in the distal colon, of which 9 were rectal adenocarcinomas.

Grading of Inflammation in *iNOS*^{+/+} and *iNOS*^{-/-} Mice

Colorectal inflammation was graded histologically in non-cancerous mucosa based on lesion severity, ulceration, glandular hyperproliferation, and area of inflammatory involvement, and expressed as a UC index as described previously [30,33]. The colons of Groups 1 and 2 negative control mice were morphologically normal. The results for the UC index analysis in DSS/2XFe-treated Groups 3 and 4 mice are summarized in Table 2. In both groups, inflammation was mild in the proximal colon, where increased mucosal thickness was observed, but ulceration and active inflammation (erosion and epithelial injury with intense inflammatory cell infiltration) were rare. UC was moderately severe in the middle and distal colon, with extensive areas of inflammation, cellular injury, and erosion, and a moderately intense inflammatory infiltrate of lymphocytes and macrophages. Hyperproliferation (characterized by decreased goblet cell numbers and elongated, branched glands often containing hyperchromatic cells) was commonly observed in these regions. The UC indices in Group 4 did not differ significantly from those of Group 3 in any of the three colon regions, or in the colon overall.

Inflammatory Cell Infiltration in the Colon as Detected by CD45 and Mac3-Immunostaining

Immunostaining for CD45 (common leukocyte antigen) and Mac3 (macrophage) was performed to analyze the effect of *iNOS* deficiency on the mucosal infiltration of lymphocytes and macrophages. A moderately intense inflammatory cell infiltrate containing CD45-positive cells (mostly lymphocytes) and Mac3-positive macrophages was present in the lamina propria of Group 3 mice (Figure 2A and C, respectively). The mucosal infiltration of CD45-positive and Mac3-positive cells showed similar intensity in Group 4 mice (Figure 2B and D, respectively). Quantitation of immunostain-positive cell numbers showed that there was no significant difference in the mean number of CD45-positive cells per area of mucosa between Groups 3 and 4 (mean $\pm$ SE, 641 ± 59 vs. 745 ± 65 immunostain-positive cells/mm² mucosa). Similarly, no difference in the number of infiltrating macrophages was observed between Groups 3 and 4 (410 ± 29 vs. 413 ± 42 immunostain-positive cells/mm² mucosa).

Cell Proliferation in Colonic Epithelia and Tumor

Proliferating epithelial cells were visualized by PCNA immunostaining. The PCNA immunostaining index was quantified in non-actively inflamed epithelia, actively inflamed epithelia, and tumors. In the normal colons from negative control mice, PCNA-positive cells

occupied the lower third of the colonic glands (the crypt region). The hyperplastic glands in the colons of DSS/2XFe-treated mice showed an expansion of the crypt region and an increase in PCNA-positive cell numbers. As shown in Figure 3, Groups 3 and 4 showed similar PCNA immunostaining indices in non-actively inflamed epithelia. Epithelia in areas of active inflammation showed higher PCNA labeling indices as compared to non-actively inflamed epithelia in both groups. There was no difference between *iNOS*^{+/+} and *iNOS*^{-/-} mice in the proliferation index for actively inflamed epithelia. Similarly, PCNA immunostaining index analysis of colon tumors showed no difference between Groups 3 and 4.

vWF Immunostain-Positive Blood Vessel Density

Colonic blood vessels were visualized by immunostaining for vWF. In the non-actively inflamed mucosa of *iNOS*^{+/+} and *iNOS*^{-/-} mice treated with DSS/2XFe, vWF-positive blood vessels were located in the lamina propria near the base of the colonic glands and occasionally near the luminal surface. Actively inflamed mucosa exhibited increased blood vessel numbers at both the base of the mucosa and near the mucosal surface. Vessels immunostain-positive for vWF were frequently observed in the tumor stroma as well. Figure 4 shows the results for the quantitation of vWF-positive blood vessel number per area of mucosa or tumor. Blood vessel density increased significantly from non-actively inflamed mucosa to actively inflamed mucosa and tumors in both Groups 3 and 4 ($P < 0.05$, one-way ANOVA test). The vWF-positive blood vessel densities in non-actively inflamed mucosa, actively inflamed mucosa, and tumors were lower in Group 4 *iNOS*^{-/-} mice as compared to Group 3 *iNOS*^{+/+} mice, with the difference reaching statistical significance in the actively inflamed mucosa (mean $\pm$ SE, 126.9 ± 1.9 vs. 103.0 ± 3.1 vWF immunostain-positive vessels/mm², $P < 0.05$).

Nitrotyrosine Formation and eNOS Expression in the Colons of DSS/2XFe-Treated *iNOS*^{+/+} and *iNOS*^{-/-} Mice

Nitrotyrosine was detected immunohistochemically to assess peroxynitrite-mediated protein modification in *iNOS*^{+/+} and *iNOS*^{-/-} mice treated with DSS/2XFe for 15 cycles. Nitrotyrosine-positive staining was frequently observed in inflammatory cells in the lamina propria, and occasionally in adjacent epithelial cells, in the actively inflamed mucosa of Group 3 *iNOS*^{+/+} mice (Figure 5A). Similar staining intensity of nitrotyrosine-positive cells was seen in Group 4 *iNOS*^{-/-} mice (Figure 5B). Numerous nitrotyrosine-positive inflammatory cells were present in the tumor stroma of mice from both Groups 3 and 4 (Figure 5C and D, respectively).

To explore the possibility that another NOS isoform may be responsible for nitrotyrosine formation in the *iNOS*^{-/-} mouse, the immunohistochemical expression of eNOS was assessed. Staining for eNOS in the colons of Groups 1 and 2 mice showed eNOS-positive blood vessels in the lamina propria of the colonic mucosa and in the submucosa (Figure 6A and B, respectively). In DSS/2XFe-treated Group 3 mice, small eNOS-positive vessels were frequently observed within the lamina propria at the surface of the mucosa in areas of active inflammation (Figure 6C), and larger eNOS-positive blood vessels were visible at the base of the colonic mucosa and in the submucosa (Figure 6E). Similarly, Group 4 mice exhibited eNOS immunostaining of small, peripheral microvessels and larger basal blood vessels (Figure 6D and F, respectively). In addition to the expected eNOS staining of blood vessels, numerous inflammatory cells in the colonic mucosa were also positively stained for eNOS, particularly in the DSS/2XFe-treated mice of Groups 3 and 4 (Figure 6G and H, respectively).

In order to study colonic eNOS protein levels in *iNOS*-deficient mice with UC, a short-term experiment was performed. eNOS protein levels were analyzed by Western blot in the colons of mice subjected to one DSS cycle and sacrificed on Day 17. A basal level of the eNOS protein (140 kDa) was observed in the colons from *iNOS*^{+/+} and *iNOS*^{-/-} negative control mice. In

contrast, the iNOS protein was not detectable in the non-inflamed colons of the negative controls (Figure 7). The levels of colonic eNOS were increased in both *iNOS*^{+/+} and *iNOS*^{-/-} mice treated with DSS/2XFe (mean band densities in arbitrary units: 0.469 ± 0.167 and 0.382 ± 0.105 , respectively) as compared to the *iNOS*^{+/+} and *iNOS*^{-/-} negative controls (band densities: 0.023 ± 0.006 and 0.032 ± 0.008 , respectively). However, there was no significant difference in eNOS protein expression between DSS/2XFe-treated *iNOS*^{+/+} and *iNOS*^{-/-} mice. As expected, iNOS protein expression was induced in the colons of *iNOS*^{+/+} mice, but not the *iNOS*^{-/-} mice, treated with DSS/2XFe (Figure 7).

The UC index was also analyzed after one cycle of DSS treatment. Inflammatory injury and hyperproliferation in the colon were less severe after 1 DSS cycle as compared to 15 DSS cycles, as indicated by lower histological scores (Table 3). There were no significant differences between *iNOS*^{+/+} and *iNOS*^{-/-} mice in any of the histological parameters of inflammation, or in the UC index overall (mean $\pm$ SE, 5.28 ± 0.31 vs. 5.30 ± 0.41), after one DSS cycle.

DISCUSSION

Several observations have led to the suggestion that NO may take part in UC-associated carcinogenesis: (1) excessive NO is produced in the colons of UC patients because of upregulation of iNOS in phagocytic inflammatory cells; (2) NO and its reactive products have mutagenic activities in vitro through both nitrosative and oxidative reactions, and NO inhibits the functions of DNA repair enzymes in vitro; and (3) NOS is upregulated in tumors, where NO may stimulate new blood vessel growth [11,18,20,21]. The present study tested the effect of *iNOS*-deficiency on UC-associated colorectal cancer development in the DSS/2XFe model in mice. The results showed that mice lacking iNOS gene expression did not differ from wild-type control mice in terms of colorectal tumor development in association with chronic UC. However, the presence of nitrosative protein modification (as detected by nitrotyrosine formation) in the DSS-induced chronic UC in iNOS deficient mice suggests the involvement of other NOS isoforms.

Depending on the model system used, inhibition or lack of *iNOS* has been reported to exacerbate, ameliorate, or have no effect on experimental colitis [34–43]. In this study, *iNOS* deficiency did not significantly affect the colonic inflammatory process induced by DSS/2XFe. The role of iNOS is probably dependent on the stage of inflammation [38,44]. For example, *iNOS* deficiency was shown to exacerbate the acute phase of TNBS-induced colitis, but had no effect on the chronic phase similar to this study using the DSS model [44]. Although there was not a marked difference in the colonic inflammation scores between the wild type and *iNOS*^{-/-} mice in this study, 6 of the 25 DSS/2XFe-treated *iNOS* knockout mice in the long-term study (vs. 2 out of 25 *iNOS*^{+/+} mice) succumbed to severe UC. Deficiency of iNOS expression may exacerbate UC, possibly because of roles of NO in angiogenesis and wound repair. As was observed in this study, *iNOS*^{-/-} mice showed decreased new blood vessel formation in actively inflamed mucosa, supporting the idea that NO takes part in the modulation of angiogenesis. Several pharmacological inhibitors of iNOS have shown inhibitory effects in various inflammation models. However, many of these agents have been shown to be relatively non-selective NOS inhibitors in vivo, suggesting that their effects may not be mediated through iNOS inhibition alone.

The effects of *iNOS* deficiency or inhibition on experimental carcinogenesis are also varied [45–47]. The NOS inhibitor aminoguanidine and L-arginine deficiency have been shown to attenuate intestinal tumorigenesis in the *Apc*^{Min/+} mouse [46]. Inducible NOS-specific inhibitors are effective in attenuating AOM-induced colon carcinogenesis [45]. *Apc*^{Min/+} mice also lacking iNOS exhibited decreased intestinal tumor development as well [46], and *iNOS*-

deficient mice are resistant to carcinogen-induced lung tumorigenesis [48]. However, another study reported that *Apc^{Min/+}/iNOS^{-/-}* mice exhibited a slight enhancement of intestinal tumor development [47]. Inducible NOS-deficient mice were just as susceptible as wild-type mice to UC-associated carcinogenesis in the present study. However, the colons of *iNOS^{-/-}* mice still exhibited nitrotyrosine staining, a marker of peroxynitrite-mediated protein modification. The retention of peroxynitrite-modified proteins in the absence of iNOS expression may be suggestive of redundancy within the NO generation pathway in activated phagocytes.

The three isoforms of *NOS* that have been characterized to date are highly homologous and are now known to be present in several cell types [13]. For example, eNOS is expressed in cardiac myocytes and blood platelets, as well as endothelial cells [49]. The observed expression of eNOS by lamina propria macrophages in both *iNOS^{+/+}* and *iNOS^{-/-}* mice in our study is in agreement with previous observations in mice and humans [50,51]. eNOS and nNOS were originally classified as constitutively expressed, calcium-dependent enzymes, whereas iNOS is calcium-independent and inducible at the transcriptional level by pro-inflammatory cytokines. The expressions of eNOS and nNOS, however, are also regulated in certain contexts. For example, eNOS is induced in vitro by hydrogen peroxide, epidermal growth factor, and transforming growth factor- β , among other stimuli [49,52]. eNOS may be a source of NO production and protein nitrosation during DSS-induced inflammation. Indeed, colonic eNOS protein levels were increased in DSS/2XFe-treated *iNOS^{+/+}* and *iNOS^{-/-}* mice. Whether or not the observed increase in eNOS was because of gene induction in activated macrophages is not known. The control of *NOS* expression is dependent on the cell type as well as the cell stimulus [52,53]. Macrophage eNOS has not been well studied, and whether it is responsive to the same stimuli as the enzyme in endothelial cells, or iNOS, is unknown.

While the cytotoxic and mutagenic effects of NO presumably occur at higher concentrations, at lower concentrations NO functions as a cell signaling molecule [11], taking part in the regulation of vascular tone and new blood vessel formation [54–58]. NO is thought to contribute to tumor progression by stimulating endothelial cell proliferation and increased blood vessel permeability, thereby facilitating neo-vascularization of the growing tumor. The critical *NOS* expressing cells and NOS isozymes involved in these processes are uncertain. Depending on the study, both tumor cells and endothelial cells in the tumor stroma have shown expression of one or more NOS isoform [59–64]. In addition, macrophages in the tumor stroma, or in the adjacent inflamed mucosa in the case of UC, may also be sources of NO that takes part in angiogenic stimulation. Inducible NOS gene deficiency did not affect tumor blood vessel density or tumor size in the present study. The mucinous tumors in this UC-associated carcinogenesis model do not appear to express iNOS either in tumor cells or intra-tumor blood vessels. However, iNOS is expressed by tumor-infiltrating macrophages, and eNOS is expressed by blood vessels and macrophages. Angiogenesis also plays a key role in the inflammatory process and wound repair [54]. Similar to tumor neo-vascularization, inflammation-driven angiogenesis may involve different cell types expressing iNOS, eNOS, or both. Based on our findings, iNOS appears to be necessary for active inflammation-driven blood vessel formation, a function that eNOS cannot totally support. The role of iNOS in wound repair, and the cell types involved in this function of iNOS, require further characterization.

Multiple NOS isoforms, including iNOS, may function together under chronic inflammatory conditions to initiate and promote carcinogenesis. It would be interesting to assess the effect of inhibiting multiple NO-producing enzymes on experimental UC-associated carcinogenesis. This could be accomplished through the use of mice harboring compound NOS gene deficiencies, or the use of general NOS inhibitors. The involvement of NOS isoforms with overlapping functions in inflammation would suggest that the use of isoform-specific pharmacological inhibitors may be insufficient to block colorectal carcinogenesis in UC patients. A multiple inhibitor or general NOS inhibitor strategy may be required. However, the

normal physiological functions of the NOS proteins, such as in wound healing, must be taken into consideration. The effects of iNOS and eNOS inhibition on ulcer healing in UC patients, for example, are unknown. Further studies in animal models are needed to clarify these issues.

Acknowledgments

The author thank Dr. Chung S. Yang for his support and encouragement. This work was supported by a NIH grant CA104741 and a research grant from the American Cancer Society to G.-Y.Y. (#RPG-00-034-01-CNE). D.N.S. was supported by a predoctoral fellowship from the New Jersey Commission on Cancer Research. C.S.Y. and G.-Y.Y. are members of the Environmental Health Center of New Jersey and the Cancer Institute of New Jersey, which made shared facilities available for this study (Center Grant ES0052).

Abbreviations

DSS	dextran sulfate sodium
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
NO	nitric oxide
PCNA	proliferating cell nuclear antigen
RONs	reactive oxygen and nitrogen species
UC	ulcerative colitis
vWF	von Willebrand Factor

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Figure 1.

Representative micrographs showing the histopathology of DSS/2XFe-induced UC and associated carcinogenesis in *iNOS*^{+/+} and *iNOS*^{-/-} mice. (A) Normal colonic mucosa in a negative mice. (B) Mild hyperplastic change with a few branching glands and small inflammatory cell infiltrates in a mouse with DSS/2XFe-induced chronic UC (125 ×). (C, D) Micrographs showing actively inflamed mucosa with epithelial injury, focal loss of epithelial glands, epithelial regeneration, and abundant infiltrating inflammatory cells (125 ×). (E) Low magnification of an invasive mucinous adenocarcinoma (25 ×). (F) Higher magnification micrograph of the adenocarcinoma in (E), showing mucin lakes and invasion into the muscle layer (125 ×). (G, H) An example of a tubular, well-differentiated adenocarcinoma with a

polypoid growth pattern ($50\times$ and $125\times$, respectively). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2.

Inflammatory cell infiltration in DSS/2XFe-treated *iNOS*^{+/+} and *iNOS*^{-/-} mice. An intense infiltration of CD45-positive inflammatory cells into the colonic mucosa was observed in *iNOS*^{+/+} (A) and *iNOS*^{-/-} (B) mice (original magnifications: 125×). Numerous Mac-3 immunostain-positive macrophages in the lamina propria of the colonic mucosa in *iNOS*^{+/+} (C) and *iNOS*^{-/-} (D) mice (125 ×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 3.

The effect of iNOS deficiency on epithelial and tumor cell proliferation in DSS/2XFe-treated mice. The PCNA labeling index was determined in non-actively inflamed mucosa of the distal colon, actively inflamed mucosa of the distal colon, and in colonic tumors of *iNOS*^{+/+} and *iNOS*^{-/-} mice subjected to 15 DSS cycles. The error bars represent SE ($n = 6$ mice per group).

Figure 4.

The effect of iNOS deficiency on vWF-positive blood vessel density in DSS/2XFe-treated mice. vWF-immunostain positive blood vessels were counted in non-actively inflamed mucosa and actively inflamed mucosa in the distal colon, and in the tumor stroma of *iNOS*^{+/+} and *iNOS*^{-/-} mice subjected to 15 DSS cycles, as described in Materials and Methods. The data are expressed as the mean number of vWF-positive vessels per mm² mucosa or tumor. Error bars represent SE ($n = 5$ mice per group). *Statistically significant difference from actively inflamed mucosa of *iNOS*^{+/+} mice, $P < 0.05$ (Student's *t*-test).

Figure 5.

Nitrotyrosine immunostaining in the colons of *iNOS*^{+/+} and *iNOS*^{-/-} mice. Nitrotyrosine immunostain-positive cells were observed in the actively inflamed mucosa (A) and tumor stroma (B) of *iNOS*^{+/+} mice treated with DSS/2XFe for 15 cycles (original magnifications: 250 ×). A similar pattern of nitrotyrosine staining was observed in the actively inflamed mucosa (C) and tumors (D) from *iNOS*^{-/-} mice (original magnifications: 125 ×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6.

Immunohistochemical expression of eNOS in the colons of *iNOS*^{+/+} and *iNOS*^{-/-} mice. eNOS immunostaining was observed in blood vessels (arrows) in the colonic lamina propria of *iNOS*^{+/+} (A) and *iNOS*^{-/-} (B) negative control mice (original magnifications: 125 ×). In the colons of *iNOS*^{+/+} and *iNOS*^{-/-} mice subjected to 15 DSS cycles, endothelial cell staining of eNOS was observed in numerous superficial blood vessels (C, D, respectively) and larger basal vessels (E, F, respectively) in the actively inflamed colon (original magnifications: 250 ×). Numerous eNOS-positive macrophages (arrows) were observed in the lamina propria of DSS/2XFe-treated *iNOS*^{+/+} (G) and *iNOS*^{-/-} (H) mice (original magnifications: 500 ×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 7.

Western blot analysis of eNOS and iNOS protein levels in the colons of *iNOS*^{+/+} and *iNOS*^{-/-} mice. *iNOS*^{+/+} and *iNOS*^{-/-} mice were treated with water or DSS and twofold iron diet for one DSS cycle, as described in Materials and Methods. Twenty-five micrograms protein from colon homogenates were loaded per lane, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. eNOS (140 kDa) and iNOS (130 kDa) were detected using isoform-specific polyclonal antibodies. Results are representative of three to four samples per group. Positive controls: lysate from human endothelial cell line for eNOS, lysate from lipopolysaccharide-stimulated RAW264.7 macrophage cell line for iNOS.

Table 1UC-Associated Colorectal Tumor Development in *iNOS*^{+/+} and *iNOS*^{-/-} Mice

Genotype	Total no. of mice	No. of tumor-bearing mice (tumor incidence) ^a	Tumor multiplicity ^b	Tumor volume ^c (cm ³)
<i>iNOS</i> ^{+/+}	23	15 (65.2%)	1.5 30.2	0.030 30.01
<i>iNOS</i> ^{-/-}	19	13 (68.4%)	2.0 30.2 ^d	0.031 30.01

^a Tumor incidence = (number of tumor-bearing mice/total number of mice per group) × 100.

^b Tumor multiplicity = number of tumors/tumor-bearing mouse. Data are expressed as mean ± SE.

^c Tumor volume = $4/3\pi r^3$; r = average tumor radius derived from three perpendicular diameter measurements. Data are expressed as mean ± SE.

^d $P = 0.05$ (Mann–Whitney Rank Sum Test).

Table 2
UC Index in *iNOS*^{+/+} and *iNOS*^{-/-} Mice: 15 DSS Cycles

Genotype	Colon region	Histological score			
		Severity	Ulceration	Hyperplasia	Area involved
<i>iNOS</i> ^{+/+}	Proximal	1.00 ± 0.00	0.33 ± 0.21	1.17 ± 0.31	2.17 ± 0.17
	Middle	2.00 ± 0.00	0.50 ± 0.22	2.00 ± 0.26	3.83 ± 0.17
	Distal	1.92 ± 0.08	1.00 ± 0.00	1.92 ± 0.33	3.83 ± 0.17
	Whole colon	1.64 ± 0.03	0.61 ± 0.10	1.70 ± 0.16	3.28 ± 0.13
<i>iNOS</i> ^{-/-}	Proximal	1.17 ± 0.17	0.17 ± 0.17	1.00 ± 0.00	2.17 ± 0.17
	Middle	2.25 ± 0.17	0.67 ± 0.49	1.67 ± 0.21	4.00 ± 0.00
	Distal	2.00 ± 0.00	0.83 ± 0.17	2.00 ± 0.26	4.00 ± 0.00
	Whole colon	1.81 ± 0.06	0.55 ± 0.17	1.56 ± 0.14	3.38 ± 0.06

Data are expressed as the mean ± SE. The mice were subjected to 15 cycles of DSS treatment as described in Materials and Methods. UC was scored in non-cancerous mucosa of the proximal, middle, and distal thirds of the colon. Three H&E-stained slides per mouse from six mice per group were analyzed. The histological scores for each animal were the average of the scores for the three slides. The UC index was the sum of the individual scores for disease severity, ulceration, glandular hyperplasia, and area of inflammatory involvement. The criteria used for the histological scoring of UC are described in Materials and Methods.

Table 3
UC Index in *iNOS*^{+/+} and *iNOS*^{-/-} Mice: One DSS Cycle

Genotype	Colon region	Histological score			
		Severity	Ulceration	Hyperplasia	Area involved
<i>iNOS</i> ^{+/+}	Proximal	0.70 ± 0.09	0.00 ± 0.00	0.20 ± 0.20	1.00 ± 0.00
	Middle	1.85 ± 0.17	0.60 ± 0.24	1.55 ± 0.20	3.70 ± 0.20
	Distal	1.35 ± 0.10	0.30 ± 0.12	1.00 ± 0.27	3.60 ± 0.19
	Whole colon	1.30 ± 0.07	0.30 ± 0.10	0.92 ± 0.16	2.77 ± 0.11
<i>iNOS</i> ^{-/-}	Proximal	0.50 ± 0.21	0.10 ± 0.10	0.45 ± 0.17	0.70 ± 0.20
	Middle	1.65 ± 0.10	0.30 ± 0.20	1.80 ± 0.22	3.50 ± 0.22
	Distal	1.50 ± 0.23	0.50 ± 0.22	1.40 ± 0.29	3.50 ± 0.22
	Whole colon	1.22 ± 0.15	0.30 ± 0.10	1.22 ± 0.14	2.57 ± 0.09
					UC index
					1.90 ± 0.23
					7.65 ± 0.48
					6.25 ± 0.58
					5.28 ± 0.31
					1.75 ± 0.58
					7.25 ± 0.56
					6.90 ± 0.92
					5.30 ± 0.41

Data are expressed as the mean ± SE. The mice were subjected to one cycle of DSS treatment as described in Materials and Methods. UC was scored in the proximal, middle, and distal thirds of the colons from mice sacrificed on Day 17. Three H&E-stained slides per mouse from five mice per group were analyzed. The histological scores for each animal were the average of scores for the three slides. The UC index was the sum of the individual scores for disease severity, ulceration, glandular hyperplasia, and area of inflammatory involvement. The criteria used for the histological scoring of UC are described in Materials and Methods.