Induction of inducible nitric oxide synthase: a protective mechanism in colitis-induced adenocarcinoma

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A close association between inflammatory bowel disease (IBD) and increased risk of developing adenocarcinoma exists. Moreover, chronic induction of high levels of nitric oxide (NO) produced from inducible nitric oxide synthase (iNOS) is a consistent observation in IBD. In this study we made interleukin-10-inducible nitric oxide synthase double-deficient (IL-10+/iNOS−/−) mice and studied the development of adenocarcinoma. Mice >6 months of age were compared with healthy wild-type (WT) controls. Inflammation was assessed using macroscopic/histological scores and myeloperoxidase activity as an indication of granulocyte infiltration. Mucosal polyps were scored macroscopically and histologically in IL-10+/iNOS−/− mice. Influenza A (H1N1) virus infection was found to significantly increase the number of polyps in IL-10+/iNOS−/− mice compared with IL-10+/iNOS+/− mice. This increase was not significantly different from WT mice; however, significantly higher numbers of polyps were observed in the absence of iNOS (P < 0.05). Hyperperfusion was noted in both groups. Signs of dysplasia and submucosal invasion were significantly higher in IL-10+/iNOS−/− compared with IL-10+/iNOS+/− mice (P < 0.05). No significant increase in p53 and β-catenin mRNA levels was observed in IL-10+/iNOS−/− over WT mice; however, a 2-fold (P = 0.06) and 3-fold (P < 0.05) increase, respectively, was noted in IL-10+/iNOS+/− mice. Our data suggest exposure to chronic NO limits abnormal p53 and β-catenin expression and reduces incidence of adenocarcinoma in IL-10+/iNOS−/− mice.

Introduction

Inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn’s disease (CD) are chronic inflammatory conditions which are associated with increased risk in developing cancer of the colon and rectum (1,2). In a large population-based study, patients with UC had a standardized incidence ratio for colorectal cancer of 5.7 and an earlier age of onset than the general population (1). Colitis-associated cancer develops through a neoplastic process detected histologically as dysplasia and it is thought that soluble mediators, such as nitric oxide (NO), generated chronically in the inflammatory milieu drive this process (3–5). It is well established that the high quantities (μM) of the free radical NO is associated with colitis (6–9) and is produced via the inducible form of nitric oxide synthase (iNOS); however, to date, a systematic study examining the causal relationship between the role of chronically produced NO and colitis-associated carcinogenesis has not been completed.

The chemistry and biological activity of NO is complicated and both pro- and anti-neoplastic roles have been proposed. During inflammation, high levels of reactive oxygen/nitrogen species are generated resulting in oxidative and nitrosative stress. In this environment, NO can generate strong reactive nitrogen species such as peroxynitrite which can cause DNA damage and form carcinogenic nitrosamines resulting in mutagenesis (10,11). It has been suggested that increased cancer incidence in chronic UC is due to mutations in, or inactivation of, key genes such as the tumor suppressor gene p53 (12) or β-catenin, a key mediator of Wnt-signaling pathway (13). Elevated iNOS activity has been shown in human colon cancer (14,15); however, there are conflicting reports on the expression of iNOS within tumor cells themselves (4,16). In contrast to the pro-neoplastic role for NO, some in vitro studies have shown that macrophages can induce cell death through iNOS-generated NO in various tumor cells including colon cancer cells (17,18). In addition, high concentrations of the NO donor, S-nitrosogluthione, can also inhibit colon cancer cell growth (19).

In animal models of chemically induced intestinal carcinogenesis, NO from iNOS has been shown to both reduce and enhance tumor development. For example, in the chemically induced azoxymethane model, inhibition of iNOS significantly inhibited colon tumor incidence (20–23). However, conflicting reports have been published on the role of iNOS in the Apcmin/+ mice, which spontaneously develop multiple intestinal polyps due to a truncating mutation in the adenomatous polyposis coli gene (24). Aham and Ohshima (25) demonstrated that treatment with a selective iNOS inhibitor, an L-arginine-independent diet or the generation of Apcmin/+iNOS−/− mice, significantly reduced the size of intestinal polyps. In a separate study, Scott et al. (26) demonstrated that genetic ablation of iNOS in Apcmin/+ mice promoted intestinal tumorigenesis. These models of colon carcinogenesis are not, however, associated with chronic inflammation and the role of NO generated from iNOS in non-chemical, chronic inflammatory disease-induced carcinoma has not so far been investigated.

Interleukin-10-deficient (IL-10−/−) mice spontaneously develop chronic colitis with macroscopic and histological similarities to human colitis (8,27) and ~60% of IL-10−/− mice will develop adenocarcinoma as they age (28,29). IL-10−/− mice develop colon cancer through the dysplasia sequence, a process similar to clinical IBD-associated cancer (29). In addition, iNOS message and protein are increased specifically in the colon of these mice from 8 weeks of age and serum nitrate/nitrite (NO end product) levels increase with age (8). In this study we used interleukin-10/inducible nitric oxide synthase double-deficient (IL-10−/−/iNOS−/−) mice to examine the role of chronic exposure to NO generated from iNOS on the development of adenocarcinoma. It should be noted that we have previously documented the development of colitis in the IL-10−/−/iNOS−/− mice to 6 months and inflammation develops to a similar level and intensity as observed (macroscopically and histologically) in IL-10−/− single mutants (8). Serum nitrate/nitrite levels after 6 months are basal suggesting no compensation from other NOS isoforms, making this a very useful model to study inflammation-associated carcinoma development (8). In this study we extended our observations over 8 months and observed an increased incidence of neoplastic characteristics in the absence of iNOS potentially via increased message and protein expression of both p53 and β-catenin.

Abbreviations: BrDU, 5-bromo-2-deoxyuridine; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBD, inflammatory bowel disease; IL-10−/−, interleukin-10 deficient; IL-10+/−/iNOS−/−, interleukin-10-inducible nitric oxide synthase double deficient; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; mRNA, messenger RNA; NO, nitric oxide; PCR, polymerase chain reaction; UC, ulcerative colitis; WT, wild-type.

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Materials and methods

All mice were bred and housed at the University of Calgary in a specific pathogen-free environment on standard chow diet. Mice deficient in IL-10 on a 129Sv/Ev background (IL-10−/−), generated by gene targeting in embryonic stem cells as described previously (30), were originally obtained from Dr RN Fedorak (University of Alberta, Edmonton, Canada). IL-10−/−/iNOS−/− mice were generated from IL-10−/− mice heterozygous for iNOS (IL-10−/−/iNOS−/+) breeding pairs as described previously (8). All mice were genotyped for iNOS by polymerase chain reaction (PCR) analysis of genomic DNA purified from tail biopsies as described previously (8). Male or female double- or single-matched mice were compared directly with IL-10−/−/iNOS-competent littersmice. Mice were studied between 6 and 8 months and age- and sex-matched 129Sv/Ev mice [wild-type (WT)] were used as non-inflected controls for comparison. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and conform to the guidelines established by the Canadian Council for Animal Care.

Circulating leukocyte counts

Mice were anesthetized by intraperitoneal injection with a cocktail of 10 mg/kg xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB, Montreal, Quebec, Canada). Whole blood was obtained via cardiac puncture and total leukocyte counts were performed using a Bright-line hemocytometer (Hauser Scientific, Horsham, PA). Leukocyte differential counts were determined by examination of blood smears stained using harco-hemacolour kit (EM science, Gibbstown, NJ).

Macroscopic and microscopic assessment of colitis

The colon was excised and colonic inflammation assessed using a previously defined scoring system which includes features of clinical colitis, the presence or absence of adhesions, strictures and diarrhea (diarrhea was defined as loose, watery stool) and the bowel wall thickness in millimeters (8). Samples of colon were fixed in formalin and processed for microscopic analysis. Hematoxylin and eosin sections were scored blindly based on a semi-quantitative scoring system described previously (8) where the following features were graded: extent of destruction of normal mucosal architecture (0, normal; 1, 2 and 3, mild, moderate and extensive damage, respectively), presence and degree of cellular infiltration (0, normal; 1, 2 and 3, mild, moderate and transmural infiltration, respectively), extent of muscle thickening (0, normal; 1, 2 and 3, mild, moderate and extensive thickening, respectively), presence or absence of crypt abscesses (0, absent; 1, present) and the presence or absence of goblet cell depletion (0, absent; 1, present). The scores for each feature were summed with a maximum possible score of 11.

Macrosopic analysis of mucosal polyp formation

Polyp formation was assessed macroscopically and scored (0–3) according to the number of polyps observed (0, none; 1, one to three individual polyps; 2, four or less individual polyps; 3, merged polyps/raised plaques). The microwave was performed by immunostaining sections with primary goat anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) and biotinylated secondary anti-goat IgG (Vector Laboratories; 1:500). Sections were labeled with Vector Elite Vectastain ABC reagents, Norwell, MA; 1:500) and biotinylated secondary anti-rabbit IgG (Vector Laboratories). Immunostaining was achieved using a Brightline hemocytometer (Hauser Scientific, Horsham, PA).

Histological analysis of neoplastic characteristics.

Neoplasia was determined histologically using a standard scoring system based on the following four criteria: crypts (0, normal; 1, goblet cell depletion; 2, branching, irregular, dilate lumen or back-to-back glands; 3, complex budding), epithelium (0, normal; 1, hyperplasia or aberrant crypt foci; 2, low-grade dysplasia: nuclear enlarge, mild hyperchromatism, nuclear crowding with stratification; 3, high-grade dysplasia: nuclear stratification, prominent hyperchromatism, pleomorphism, loss of nuclear polarity), crypt crowding and cibirifining, submucosal invasion (0, absent; 1, present) and serosal adhesions (0, absent; 1, present). As a negative control, duplicate sections were immunostained with modified Harris hematoxylin. β-Catenin immunostaining was achieved by incubating sections with primary goat anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) followed by peroxidase-blocking solution (3% H2O2 in phosphate-buffered saline) and biotinylated rabbit anti-goat IgG (Vector Laboratories; 1:500). Sections were incubated with horseradish peroxidase–streptavidin (Vecto Laboratories) and DAB peroxidase and counterstained with Gill’s hematoxylin.

Determination of tissue myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured as a biochemical marker of granulocyte infiltration into tissue (31). MPO activity was assessed in samples of colon and lung tissue as an indication of local and systemic inflammation, respectively. Tissue samples were frozen on dry ice and stored at −20°C for no more than 1 month before the MPO activity was performed as described previously (7,8). The rate of change in absorbance at 450 nm over a 90 s period was determined using a kinetic microplate reader ( Molecular Devices, Sunnyvale, CA, USA). One unit of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide per minute at 25°C. Values are expressed as units of MPO activity per milligram of tissue sampled (U/mg tissue).

Quantification of p53 and β-catenin messenger RNA levels by real-time PCR

Total RNA was isolated from ascending colon of WT, IL-10−/− and IL-10−/−/iNOS−/− mice, using the QIAzol solution (Qiagen Science, Mississauga, ON) and the genomic DNA cleaned using the Deoxyribonuclease Message Clean kits using protocols as suggested by the supplier (GenHunter Corporation, Brookline, MA). Total RNA was quantified by measuring the absorbance at 260 nm. Complementary DNA (cDNA) was synthesized and amplified using standard reverse transcription and PCR procedures (32).

Real-time PCR.

The primers and probes for p53 and β-catenin were designed using the Primer Express software (PE Applied Biosystems, Foster City, CA) and based on sequences reported by the ENSEMBL database. The oligonucleotide and probe sequences used are listed in Table I.

Triple samples of 5 μl of each cDNA (1:5 diluted) were amplified by PCR in the ABI Prism 7000 Sequence Detection System (PE Applied Biosysstem). The amplification reaction mixture (25 μl) contained 2 μl of cDNA, 2.25 μl of forward primers, 2.25 μl of reverse primers (final concentration 900 nM/μl, Sigma, Oakville, ON), 0.5 μl of probe (final concentration 200 nM/μl, Sigma) and 2× Universal Master Mix (Applied Biosystems, Streetsville, ON). GAPDH was co-amplified as an internal control to normalize for variation amounts of cDNA in each sample, using 20× GAPDH Mix (Applied Biosystems). The thermocycler parameters were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were collected and analyzed to determine the PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, Ct). 2 SDs above the mean fluorescence generated during the baseline cycles. A comparative Ct method was used to measure relative gene expression. Treated samples (X) and the control sample (Y) are normalized to endogenous reference gene GAPDH. Relative expression equals 2ΔΔCt, where ΔΔCt = ΔCt(X) − ΔCt(Y) and ΔCt is the difference in target Ct and GAPDH Ct.

p53 and β-catenin protein expression by immunohistochemistry

Immunohistochemical analysis for p53 and β-catenin was performed on 5 μm thick, formalin-fixed, paraffin-embedded tissue sections from the same area of ascending colon in WT, IL-10−/− and IL-10−/−/iNOS−/− mice as indicated for hematoxylin and eosin sections. Sections were deparaffinized in xylene and rehydrated to phosphate-buffered saline before immunohistochemical processing as described previously. Briefly, p53 immunostaining was achieved using avidin/biotin blocking kit (Vector Laboratories, Burlington, ON) followed by incubating sections with primary rabbit anti-p53 (CM5, Novacastro Laboratories, Norwell, MA; 1:500) and biotinylated secondary anti-rabbit IgG (Vector Laboratories; 1:500). Sections were labeled with Vector Elite Vectastain ABC (Vector Laboratories), incubated with 3,3′-diaminobenzidine (DAB) chromagen and counterstained with modified Harris hematoxylin. β-Catenin immunostaining was achieved by incubating sections with primary goat anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) followed by peroxidase-blocking solution (3% H2O2 in phosphate-buffered saline) and biotinylated rabbit anti-goat IgG (Vector Laboratories; 1:500). Sections were incubated with horseradish peroxidase–streptavidin (Vecto Laboratories) and DAB peroxidase and counterstained with Gill’s hematoxylin.

As a negative control, duplicate sections were immunostained without exposure to the primary antibody. For p53 analysis, sections from each sample were analyzed (×40) and the percentage of brown-stained epithelial cell nuclei was calculated as a total of the epithelial cells. Positive staining was taken as >10% brown-stained epithelial cell nuclei per sample. For β-catenin staining, sections were studied for the presence of staining along cell–cell junctions, in the cytoplasm and for translocation to the nucleus.

5-Bromo-2-deoxyuridine analysis

In a separate group of mice, 5-bromo-2-deoxyuridine (BrDU) incorporation into cells entering mitosis (S-phase) was used to detect the proliferating cell population. Mice received an intraperitoneal injection of 100 mg/kg of BrDU 1 h prior to killing. Animals were killed between 9 and 12 a.m. to control for the circadian rhythm that affects cell division.

Table I. The primers and probes of p53 and β-catenin

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<tr>
<th>Primer/Probe</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Probes sequence</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>p53 forward primer</td>
<td>ACTTTGAGAGATTTCA</td>
<td>GAAGCACGCTGTCGTTG</td>
<td>5′-CCTCGAGTCTTGTC-3′</td>
<td>[DFAM][CCCTACAGTCGCCCGGCGGTTA]</td>
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<tr>
<td>p53 reverse primer</td>
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<td>[AC]</td>
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<td>[DFAM][CAGTGGCCCTCTGATAAAGG-CAACTGTGGG[DFAM]</td>
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diurnal variation in cell cycling. Samples of colon tissue were taken from approximately the same region of ascending colon in all mice and prepared for histological analysis. BrdU-positive staining was determined by immunohistochemistry as described previously. Briefly, non-specific binding was blocked on deparaffinized sections and incubated with primary rat anti-BrdU (Jackson ImmunoResearch, West Grove, PA; 1:1000). Four (×40) microscopic fields from each section were photographed and epithelial cells positive for BrdU staining counted and expressed per square micrometer.

**Statistical analysis**

Data are expressed as the mean ± standard error of the mean. Groups of data were compared using non-parametric Mann–Whitney U test or Kruskal–Wallis one-way analysis of variance followed by a Dunns multiple comparison test. Probabilities (P) of <0.05 were considered statistically significant.

**Results**

**Inflammation develops in the absence of chronic NO synthesis from iNOS**

Table II illustrates parameters of inflammation assessed in IL-10/−/−, IL-10/−/−/iNOS/−/− and WT mice between 6 and 8 months of age. Inflammation was assessed by macroscopic and microscopic scoring systems as described previously (8) and both IL-10/−/− and IL-10/−/−/iNOS/−/− mice showed similar levels of inflammation (P < 0.05 from WT scores). Granulocyte infiltration into the colon as assessed by MPO activity was significantly elevated and similar in both mutant groups. These data illustrate that inflammation develops in both the IL-10/−/− and IL-10/−/−/iNOS/−/− mice to a similar level and corroborate more detailed data previously generated in these mice (8). Relative expression of iNOS messenger RNA (mRNA), determined using real-time PCR of colon tissue, was significantly elevated in IL-10/−/− mice compared to WT mice at 6.0 ± 0.9 U/mg (iNOS) versus 2.1 ± 1.7 respectively. Although expression levels were not sectioned for determination of neoplasia, histological

**Macrosal polyps presentation is augmented in the absence of NO**

Polyp formation was isolated to the ascending colon and was located ~2–4 mm from the cecum extending distally. IL-10/−/− mice had an average polyp score of 1.1 ± 0.2 (representing one to three individual polyps) which involved ~5.9 ± 3.1 mm of colon (Figure 1A, right). Maximum polyp diameter averaged <2.5 mm in IL-10/−/− mice (mean ± standard deviation = 2.47 ± 1.0). Surprisingly, IL-10/−/−/iNOS/−/− mice had enhanced polyp scores (P < 0.01) with the majority of mice presenting with four or more polyps or merged raised masses involving almost double the length of the colon (11.4 ± 1.6 mm) (Figure 1A, left). Figure 1B details the severity and location of polyps for individual animals between the groups. The average maximum polyp diameter for IL-10/−/−/iNOS/−/− mice was 4.2 ± 2.1 (mean ± standard deviation, P < 0.05). Although individual polyps were not sectioned for determination of neoplasia, histological
analysis was performed on tissue samples taken from the ascending colon which contained mucosal hyperplasia. It is worth noting that although the number and extent of polyps differed a similar incidence in mucosal polyps was observed in IL-10−/− mice (79%) and in IL-10−/−/iNOS−/− mice (83%).

Dysplastic changes were determined histologically in a blinded fashion. Figure 2 illustrates the dysplasia score assessed in WT, IL-10−/− and IL-10−/−/iNOS−/− mice. IL-10−/− mice had a mean dysplasia score of 3.8 ± 0.6 which represented irregular crypt formation with branching or back-to-back glands (reduced stroma) and hyperplasia or aberrant crypt foci with low high-grade dysplasia. Adenocarcinoma defined as submucosal invasion of irregular crypts with signs of high-grade dysplasia was also noted in 22% of samples. IL-10−/−/iNOS−/− mice had significantly higher dysplasia scores (6.2 ± 0.6) representing abnormal crypt formation with complex budding and hyperplasia or aberrant crypt foci with high-grade dysplasia. Adenocarcinoma was identified in 61% of double-mutant samples.

Figure 3 illustrates hematoxylin and eosin sections depicting normal colon architecture from WT mice (Figure 3A) and adenocarcinoma observed in IL-10−/− (Figure 3B and C) and IL-10−/−/iNOS−/− (Figure 3D–F) mice. Figure 3B illustrates areas of normal architecture (black arrows) juxtaposed to malignant area (boxed) where mucosal dysplasia was evident and many malignant glands have invaded into the submucosa and muscle layers. (F) Higher magnification of (E) at boxed area (solid line: 100 μm bar). Adenocarcinoma, defined as submucosal invasion of neoplastic glands, was noted in 22% of IL-10−/− mice and 61% of IL-10−/−/iNOS−/− mice.
deep ulceration (Figure 3D) and active fibroblast proliferation was observed.

**Epithelial cell proliferation was observed in IL-10−/− and IL-10−/−/iNOS−/− mice**

BrdU incorporation into colonic epithelial cells was determined immunohistochemically as an indication of cell proliferation and is presented in Figure 4. BrdU-positive cells were assessed in four sections from each mouse and expressed per unit area. A significant increase in the number of BrdU-positive staining cells was observed in both IL-10−/− (P < 0.05) and IL-10−/−/iNOS−/− (P < 0.01) mice compared with WT mice. Data generated from the IL-10 mutants were not significantly different from each other. Representative immunohistographs of BrdU staining in WT, IL-10−/− and IL-10−/−/iNOS−/− mice are presented in Figure 5A, B and C, respectively. There are few BrdU-positive cells in WT mice (Figure 5A) and immunostaining is located in the lower two-thirds of each crypt. More BrdU-positive cells are observed in both the single (Figure 5B) and the double knockout (Figure 5C) mice and BrdU labeling extends to the luminal edge of the crypts.

**p53 expression is enhanced in the absence of iNOS in IL-10−/− mice**

Using real-time PCR we quantified p53 message in the colon of WT, IL-10−/− and IL-10−/−/iNOS−/− mice (Figure 6A). Relative expression of p53 mRNA in IL-10−/− mice was not significantly different from WT mice; however, a significant 2-fold difference in p53 mRNA was observed in IL-10−/−/iNOS−/− mice (P < 0.05). The difference between IL-10−/− and IL-10−/−/iNOS−/− mice did not quite reach significance (P = 0.06). Immunohistochemical analysis showed positive staining for p53 expression in many epithelial cell nuclei in IL-10−/−/iNOS−/− mice (Figure 5F), especially in areas of submucosal invasion. Very few (<10%) positive staining cells were observed in IL-10−/− mice (Figure 5E) and no p53-positive staining was observed in WT mice (Figure 5D).

**β-Catenin message and expression in the absence of iNOS in IL-10−/− mice**

β-Catenin mRNA levels were quantified in WT, IL-10−/− and IL-10−/−/iNOS−/− mice by real-time PCR and relative data expressed in Figure 6B. A significant increase in β-catenin mRNA was observed in double mutants (2.3 ± 0.7) compared with IL-10−/− (0.7 ± 0.2) and WT (0.7 ± 0.4) mice. β-Catenin mRNA levels in IL-10−/− mice were not different from WT mice. Next we measured β-catenin protein expression and localization within the cells using immunohistochemistry. β-Catenin translocation from the cell membrane to the cytoplasm or nucleus is required for increased transcriptional regulation of key target molecules involved in carcinogenesis. Figure 7 illustrates β-catenin protein expression in WT (Figure 7A and A1), IL-10−/− normal tissue (Figure 7B and B1), IL-10−/− adenocarcinoma (Figure 7C and C1) and IL-10−/−/iNOS−/− adenocarcinoma (Figure 7D and D1) mice. In normal colon epithelial cells from healthy conditions, β-catenin expression was cytoplasmic and membrane-bound, whereas in IL-10−/−/iNOS−/− mice, β-catenin expression was observed in the nucleus, leading to increased transactivation of target genes.

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**Fig. 4.** BrdU-positive cells were visualized by immunohistochemistry in sections of ascending colon from WT, IL-10−/− and IL-10−/−/iNOS−/− mice at 6–8 months of age. BrdU-positive cells were assessed in four sections from each mouse and expressed per unit area. Values are expressed as mean ± standard error of the mean from n = 5 mice. *P < 0.05 and **P < 0.01 from WT mice.

**Fig. 5.** Representative immunohistographs of ascending colon from WT, IL-10−/− and IL-10−/−/iNOS−/− mice at 6–8 months of age. Sections were stained for BrdU (A–C) or p53 (D–F) immunoreactivity. Few BrdU-positive cells were observed in WT mice (A) and immunostaining was located in the lower two-thirds of each crypt. More BrdU-positive cells are observed in the IL-10−/− (B) and the double knockout (C) mice and BrdU labeling extends to the luminal edge of the crypts. Immunohistochemical analysis of p53 expression showed that 40% of epithelial nuclei cells in IL-10−/−/iNOS−/− mice had positive staining for p53 (F), especially in areas of submucosal invasion. Very few (<10%) positive staining cells were observed in IL-10−/− mice (E) and no p53-positive staining was observed in WT mouse (D).
(Figure 7A and A1) or IL-10−/− (Figure 7B and B1) mice, β-catenin was localized at the cell–cell borders. β-Catenin staining in normal tissue from IL-10−/−/iNOS−/− mice did not differ from normal tissue in IL-10−/− mice (picture not shown). In contrast, homogeneous cytoplasmic and scattered nuclear immunostaining was observed in adenocarcinomas from either IL-10−/− (Figure 7C and C1) or IL-10−/−/iNOS−/− (Figure 7D and D1) mice. A cytoplasmic and nuclear localization of β-catenin or loss of cell membrane staining was also observed in dysplastic tissues, but hyperplastic tissue only stained for β-catenin at the cell membranes, as with normal colon epithelial cells. No difference in protein expression was noted in adenocarcinoma from either IL-10−/− or IL-10−/−/iNOS−/− mice; however, the incidence of adenocarcinoma was enhanced in the double knockouts. Negative control sections showed no positive staining when the primary antibody step was omitted.

Discussion

Patients with chronic inflammatory conditions such as UC and Crohn’s disease have an increased risk of developing colon cancer than the general population; however, to date, few studies have addressed this issue due to the lack of a suitable model of inflammation-driven intestinal cancer. In this study we used the IL-10-deficient model of spontaneously developing colitis to study the role of NO generated from iNOS in the development of inflammation-associated adenocarcinoma. The IL-10−/− model not only holds many similarities to clinical IBD, including chronically elevated levels of NO produced from iNOS (8), but also develops colitis-associated dysplasia which progresses to adenocarcinoma and is histologically similar to IBD-associated cancer in humans (28). Using IL-10−/−/iNOS−/− mice, we demonstrated that iNOS, associated with chronic inflammation of the colon, acts to limit the development of adenocarcinoma. Using IL-10−/−/iNOS−/− mice, we observed increased macroscopic polyph formation and histological characteristics of dysplasia including submucosal invasion of neoplastic crypts (61 versus 22% of IL-10−/− mice). In addition, we observed increased message and protein for tumor-associated markers p53 and β-catenin in the absence of iNOS. Our data support the view that chronic NO produced from iNOS during intestinal inflammation decreases the risk of developing intestinal cancer.

It is generally accepted that the increased risk of developing cancer associated with IBD is primarily acquired secondarily to colonic inflammation (2,3) as opposed to having a genetic predisposition (33). However, it has only recently been established that the severity of the colonic inflammation is an important factor of the risk of developing neoplastic changes (2). In this present study, we demonstrated that the severity of inflammation between our IL-10- and IL-10/iNOS-deficient groups was comparable, as assessed by macroscopic scores, histological scores and colonic tissue MPO activity and corroborated our previous findings in this model (8). These data suggest that differences observed in this study in the incidence and extent of dysplastic changes are not related to different levels of inflammation between the two groups and supports the theory that inflammatory mediators are important in the development of inflammation-associated cancer (4).

We have shown previously by reverse transcription–PCR and immunohistochemistry, respectively, that iNOS message and protein are elevated chronically in the IL-10−/− and not IL-10−/−/iNOS−/− mice (8). The end products of NO generation, nitrate/nitrates, also increased with age in the single mutant only. In this current study we corroborated our previous data using quantitative PCR to illustrate elevated message for iNOS in IL-10−/− mice and not the double knockouts. In addition, we have demonstrated that although ~80% of IL-10−/− (single or double) mutants present with macroscopic polyphs, in the absence of iNOS polyphs are increased in number and size. The development of adenocarcinoma, defined as the invasion of neoplastic cells through the muscularis mucosae, was also significantly increased in the double-mutant mice. These data suggest that lack of chronic NO production from iNOS during inflammation leads to an increased incidence of adenocarcinoma.

NO produced via iNOS, by a number of inflammatory cells (neutrophils, monocytes, macrophages) as well as epithelial cells during IBD, has been proposed to be pro-neoplastic through its ability to form reactive nitrogen species such as peroxynitrite and induce oxidative DNA damage (DNA base modifications, strand breaks and mutations) or through the formation of nitrosamines through N-nitrosation of secondary amines (34). Our data support a role for NO in limiting the development of colon cancer; it is possible that in the absence of NO a large antioxidant effect is reduced, resulting in enhanced DNA damage through reactive oxygen species.

β-Catenin is a key component of the cadherin-mediated cell–cell adhesion complex and in intracellular signaling through the Wnt-signaling pathway (13). Translocation of β-catenin from the cell membrane to the cytoplasm or nucleus is an early event in human colorectal carcinogenesis (35) and mutations in β-catenin and cytoplasmic/nuclear translocation have been reported in various chemically induced models of cancer (22,36,37). Takahashi et al. (22) demonstrated co-expression of iNOS and β-catenin alterations in azoxy-methane-induced rat colon cancer, suggesting β-catenin alterations may be related to iNOS expression. In addition, in a chemically induced (dextran sodium sulfate) model of inflammation-associated cancer, Cooper et al. (38) demonstrated translocation of β-catenin to the cytoplasm and nucleus in 100% of dysplasia-associated lesions or mass. In our present study, which is the first to study β-catenin message and protein expression in the IL-10−/− mouse model, we observed basal mRNA for β-catenin relative to WT control.
addition, using immunohistochemistry, we demonstrated enhanced cytoplasmic staining for β-catenin in areas of adenocarcinoma from either IL-10−/− or IL-10−/−/iNOS−/− mice. Interestingly, in the absence of iNOS, we observed a significant 3-fold increase in β-catenin mRNA expression. No significant differences in β-catenin staining were observed between IL-10−/− and IL-10−/−/iNOS−/− mice in areas of normal tissue or in areas of adenocarcinoma; however, increased staining associated with adenocarcinoma was observed more...
frequently in the absence of iNOS. These data suggest that alterations in β-catenin expression are associated with the increased adenocarcinoma incidence observed in the absence of iNOS. These data do tell us whether the lack of iNOS has a direct effect on the translocation of β-catenin to the cytoplasm and nucleus; however, a recent study by Lee et al. (39) demonstrated that β-catenin dissociation can be modulated by iNOS/cyclic guanosine monophosphate-dependent protein kinase activity through a direct structural association of iNOS with N-cadherin/β-catenin at cell–cell junctions.

The p53 protein is the product of the tumor suppressor gene (p53) which is increased in the presence of DNA fragmentations (40) and has been used as a marker for neoplastic changes in clinical and experimental models of colitis (41,42). Although genetic mutations in p53 gene itself can lead to colon cancer in familial polyposis for example, adenocarcinoma development in IL-10−/− mice is not linked to any genetic mutation in p53 (29). Previously, Sturlan et al. (29) observed 60% high-grade dysplasia in IL-10−/− mice on a C57Bl6 background with no overexpression in p53 by immunohistochemistry. Our present findings in IL-10−/− mice on a 129Sv/Ev background where we did not observe an increase in p53 mRNA or protein expression corroborates this previous finding. However, a 2-fold increase in p53 mRNA was observed by quantitative PCR in the IL-10−/−/iNOS−/− mice together with an increase in positive staining for p53 protein observed by immunohistochemistry (40 versus <10% in IL-10−/− mice). Taken at face value, these data support our overall findings of increased neoplastic changes in the absence of iNOS. However, several recent studies have observed that NO can phosphorylate p53 and stimulate functional p53 activity (43,44). Taken together with these recent findings, our data suggest that NO generated via iNOS in chronic IBD acts to promote functional p53 activity and suppresses the development of cancer.

In conclusion, absence of chronic NO production from iNOS resulted in increased colon cancer development in IL-10−/− mice. Alterations of β-catenin expression and overexpression of p53 were demonstrated in the absence of iNOS. Our data strongly support an anti-neoplastic role for chronic NO production in this spontaneous model of colitis.

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