Prolonged Chronic Inflammation Progresses to Dysplasia in a Novel Rat Model of Colitis-Associated Colon Cancer

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Abstract

Inflammatory bowel disease (IBD) is a gastrointestinal disorder of unknown etiology or cure. One complication of IBD is an increased risk for development of colon cancer. The aims of this study were to use a previously established rat model of colitis to develop a new model of colitis-associated colon cancer and ascertain the involvement of three cancer-related genes: K-ras, adenomatous polyposis coli (APC), and p53. Four groups of rats were used: reactivated 1,2-dimethylhydrazine [DMH]; trinitrobenzene sulfonic acid (TNBS) was used to induce colitis followed by a weekly s.c. dose of DMH], prolonged reactivation (inflammation was induced with TNBS, then maintained twice a week), saline-DMH (animals received saline instead of TNBS followed by a weekly dose of DMH), and normal (received no treatment). Animals were sacrificed at 5, 10, or 15 weeks, and colorectal samples were taken for pathologic analysis and gene mutation detection. No dysplasia was found in the normal group. The highest incidences of dysplasia were as follows: prolonged reactivation group at 5 weeks (60%), reactivated DMH group at 10 weeks (83%), and saline-DMH group at 15 weeks (67%). Carcinoma was found in both the prolonged reactivation and saline-DMH groups. No mutations were found in the K-ras oncogene; however 62% of the APC samples (exon 15 at nucleotide 2778) and 76% of p53 (exon 6 at nucleotide 1327) showed substitutions. The prolonged reactivation group may be considered a new model of colitis-associated colon cancer, offering the potential to study cancer prevention strategies for patients with IBD. [Cancer Res 2007;67(22):10766–73]

Introduction

Inflammatory bowel disease (IBD) is a debilitating disorder of unknown etiology wherein inflammation, ulceration, and bleeding are present in the gastrointestinal mucosa in conjunction with diarrhea (1). Crohn’s disease and ulcerative colitis are the most common forms of IBD, differentiated mainly by location and depth of inflammation (2).

One complication of ulcerative colitis is an increased risk for the development of colorectal cancer (3). Each year, >140,000 Americans are diagnosed with colon cancer and >55,000 die from this disease (4, 5). Similar to IBD, the causes and mechanisms of colon cancer are poorly understood. Markers that can help in the prevention or early detection of colon cancer are needed to combat the mortality associated with the disease. At the present time, the most commonly used marker for colon cancer is dysplasia (6).

Dysplasia is a cellular and structural alteration of the colon mucosa whose diagnosis requires a patient biopsy (7). However, the use of dysplasia as a prognostic marker for colon cancer has been questionable because of high interphysician variability in the diagnosis (6). An animal model that mimics the initial chronic inflammation, which progresses to dysplasia and colon cancer, would be an invaluable tool to look for more specific markers than dysplasia (8). Such a model may also offer the potential to look for less invasive markers in serum or plasma.

Several animal models have previously been proposed to study sporadic and colitis-associated colon cancer, including transgenic and immunodeficient mice including interleukin-10 (IL-10) or IL-2 knockouts (9, 10). However, in IL-10−/− mice, spontaneous inflammation may take months to develop, suggesting the presence of other regulatory factors that can delay the onset of IBD (11). Moreover, IL-2−/− mice become severely compromised after 4 weeks of age with ~50% of the animals dying between 4 and 9 weeks after birth (12). This low survival rate represents an impediment for the study of prolonged chronic colitis.

These models have also been used in combination with carcinogens, such as 1,2-dimethylhydrazine (DMH), a toxic environmental pollutant and colon-specific procarcinogen, which works through the metabolism of the alkylating ion methyldiazonium (13) or its metabolite azoxymethane (14). Irrespective of the administration route, both compounds induce tumors within the descending colon which are histologically similar to those of human sporadic colon cancer (15).

A disadvantage with many of the aforementioned models is that they do not truly mimic a colitis that then progresses to colon cancer. If a more predictive model could be developed, studying this transition will offer the ability to search for potential molecular markers, in addition to offering new methods for prevention and treatment. This is important because it has been found that patients that are initially diagnosed with colon cancer have a 5-year survival rate of 93% if diagnosed at stage 1, which decreases to only 8% if diagnosed at stage 4 (16).

A genetic model for the development of colon cancer was originally proposed by Fearon and Vogelstein (8). It was postulated that the accumulation of changes determines the biological properties of the tumors instead of the order of the events. Possible genetic changes include the inactivation of tumor suppressor genes and/or activation of oncogenes. K-ras is one of the most commonly studied oncogenes because of its activation in many tumor types. The molecular role of K-ras in cancer is still unknown because K-ras activation occurs as an early event in some types of cancer whereas it is associated with invasion and metastasis in others (17). Adenomatous polyposis coli (APC) is a tumor suppressor gene that is mutated in up to 85% of sporadic colorectal cancers and in familial adenomatous polyposis. p53, another tumor suppressor gene, is probably the most extensively studied molecule in the cancer field. More than 50% of malignant human tumors involve mutations in the TP53 gene (18).
The aims of the present study were to create a new model of colitis-associated colon cancer based on a previously well-established “reactivated” rat model of chronic colitis (19) and use this new model to look for possible alterations in the K-ras, APC, and p53 genes. The reactivated model has already been used extensively in our laboratory, as well as in others, to investigate colonic secretion (20), motility, bacteria (21, 22), and cytokine expression (23). We hypothesized that continuing the chronic inflammation would produce dysplastic and molecular changes over time.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats weighing 190 to 210 g at the start of the initial treatment were maintained in restricted-access rooms with controlled temperature (23°C) and 12-h light-dark cycle. They received standard laboratory chow and drinking water *ad libitum*. Animal protocols were approved by the Institutional Animal Care and Use Committee at Ponce School of Medicine.

**Administration of Proinflammatory and Procarcinogenic Compounds**

Four groups of animals were used: two experimental (prolonged reactivation and reactivated DMH), one control group (saline-DMH), and a normal group (normal).

Inflammation was induced in the prolonged reactivation group by installation of trinitrobenzene sulfonic acid (TNBS; 0.5 mL of 60 mg/mL; Sigma Aldrich) in 50% ethanol followed by reactivation (19). Induction was accomplished rectally via a catheter inserted – 8 cm proximal to the anus. For the reactivation, after 6 weeks, the rats were lightly anesthetized with ether, and 5 mg/kg TNBS in 0.9% saline was given i.v. via a tail vein. These i.v. injections were repeated 24 and 48 h later. One week after the last reactivation, treatment with TNBS i.v. was continued twice a week for up to 15 weeks.

The reactivated DMH group was induced and initially reactivated for colitis at 6 weeks as above. These animals then received, 1 week later, an s.c. injection of 20 mg/kg body weight of DMH (Aldrich Chemical Company). DMH was then given weekly at the same time on the same day each week for up to 15 weeks. DMH was freshly prepared as a 0.5% solution in 1 mmol/L EDTA (Sigma-Aldrich) adjusted to a pH of 7.0 with 10% sodium bicarbonate immediately before injection (24).

The control group (saline-DMH group) was induced and reactivated with saline instead of TNBS, and the animals were treated with DMH weekly thereafter as previously explained. Animals in the normal group were untreated. All animals were weighed weekly until the end of the study and sacrificed at 5, 10, or 15 weeks with an overdose of pentobarbital (– 15 mL of 65 mg/mL for rats of >500 g). The experiments reported herein were done in accordance with the principles described in the “Guide for the Care and Use of Laboratory Animals,” publication no. DHHS NIH 86-23.

**Measurement of Macroscopic Damage**

After sacrifice, the colons were removed. Four variables were examined as previously described (19): the presence of diarrhea (0 or 1 for absence or presence), adhesions between the colon and other organs (0, 1, or 2 for none, minor, or major, respectively), the thickness of each colon segment (in millimeter), and the degree of ulceration (0 for no damage; with increasing scores up to 10, depending on the extent of ulceration). These variables were added to give a total macroscopic damage score.

**Sample Collection**

The colon length was measured in centimeters and cut in equal thirds representing the proximal, mid, and distal parts of the colon. These segments were cut longitudinally; one half was weighed and stored at –20°C for molecular analysis, and the other half was fixed in 10% buffered formalin. The Swiss-roll technique was used to allow microscopic evaluation of the entire length of the intestine at once (25).

**Microscopic Assessment**

The tissues were analyzed by a blinded observer and scored microscopically for damage as previously published (19). Criteria included loss of mucosal architecture (0–3: absent, mild, to severe), cellular infiltration (0, none; 1, in muscularis mucosae; 2, in lamina propria/villi; 3, in serosa), muscle thickening (0, muscle < 1/2 of mucosal thickness; 1, muscle = 1/2 to 3/4 of mucosal thickness; 2, muscle = mucosal thickness; 3 = all muscle), goblet cell depletion (0, absent; 1, present), and crypt abscess formation (0, absent; 1, present). The score of each variable was added to give a total microscopic damage score (maximum of 11).

**Pathologic Evaluation**

Colon sections (4 μm) stained with H&E were analyzed by a blinded pathologist for neoplastic changes. Histologic analysis for dysplasia was scored based on previously published criteria (26). Briefly, tissue sections were classified as either negative for dysplasia, or positive for dysplasia or carcinoma. The tissues classified as negative for dysplasia adhered to one of the following descriptions: normal (small nucleus and normal architecture), inactive colitis, or active colitis (cryptitis, glandular invasion by neutrophils, crypt abscesses, microabscesses). A classification of positive dysplasia was characterized by low-grade dysplasia, which included hyperchromasia, increased nuclear/cytoplasmic ratio, irregular nuclear outline, and increased number of normal mitosis. The criteria for high-grade dysplasia included the characteristics of low-grade dysplasia plus loss of mucosal architecture in the crypts. For a diagnosis of carcinoma, the characteristics of high-grade dysplasia were included, but instead of an increased number of normal mitosis, an increased number of atypical mitosis was observed.

**PCR Amplification**

DNA for PCR amplification was extracted using the QIAamp DNA minikit (Qiagen) followed by ethanol precipitation for concentration and salt removal. PCR amplification of the K-ras gene exon 1 that contains codons 12 and 13 was done in a volume of 50 μL using the Optimase Polymerase deoxynucleotide triphosphate kit (Transgenicom, Inc.) and the primers F 5’-GGATCCCGCTGTAAAAAGCTGAGTA, R 5’-GGTTCCTGGCTCAGGATAATTTCTATCC. The 117-bp product was confirmed by agarose electrophoresis. PCR for APC gene exon 15, covering a 2,738-bp region, including the region designated as the mutation cluster region in human colorectal cancer was amplified using three pairs of primers that generated three overlapping segments spanning exon 15. The primer sequences and PCR conditions have been described elsewhere (27). PCR amplification was verified by gel electrophoresis with a 0.8% agarose in 1× TAE buffer. The band size was 987 bp for segment A, 1,062 bp for segment B, and 925 bp for segment C. The majority of p53 mutations in human colorectal cancer occur within a highly conserved area spanning codons 110 to 307; therefore, exons 6 and 7 of the p53 gene were amplified using primer sequences published elsewhere (27).

**Mutation Detection**

K-ras exon 1. Mutations at codons 12 and 13 of the K-ras gene were detected using denaturing high-performance liquid chromatography (DHPLC) analysis (Transgenomic WAVE MD System; Transgenomic). The DHPLC system was used as a screen to identify samples with possible mutations in this gene. The hybridization was done in a mastercycler, and the conditions consisted of 95°C for 5 min, followed by a step decrease in temperature of 0.5°C for 15 s per step until the temperature reached 45°C (99 more cycles). This reaction contained 10 μL of experimental PCR product (possible mutant) combined with 10 μL of PCR product from a known normal rat. The hybridization protocol produced homoduplex (nonmutated) and/or heteroduplex (mutated) products that were run in the DHPLC system for visualization in a chromatogram.

Cloning and sequencing procedures were done only in those samples that presented a different pattern in comparison with the reference on the WAVE analysis. The sequencing reaction was done using a SequiTHERM EXCEL II Labeled Primer Sequencing Kit-1C (Epigenic Biotechnologies) following the manufacturer's recommendations, and the analysis was done using the BioEdit freeware software (BioEdit V7.0; Tom Hall).
**APC and p53.** To look for mutations in the APC and p53 genes, we chose representative samples based on the pathologic diagnosis, because the product of the three segments of the APC gene was too large to be prescreened using the WAVE system and the PCR of the p53 gene was done using the Taq polymerase, which is not compatible with the WAVE MD system.

Detection in relevant sections was done by direct sequencing as follows: PCR products were purified using the QIAquick Spin columns (Qiagen). For the sequencing reaction, we used 20 ng of template for the APC gene and 5 ng of template for the p53. The total volume for the reaction was 12 μL:11 μL for the template and 1 μL of the sequencing primer (3.2 pmol/μL). Nested primers used for the sequencing reactions were as follows: APC A segment, forward 5′-AATCTCTACGGAGAAATCC; APC B segment, forward 5′-TGG-CAATGTTAAGGGG/reverse 5′-TCTGCTCCTCCTGTTTGC; APC C segment, forward 5′-CATCGCTAATCATCAGCTGAGG; and p53 (6, 7), forward 5′-CCTGACTATTCTTGCTC. Samples were sequenced, and analysis was done as described above.

**Statistical Analysis**

Values are presented as mean ± SE, wherein n represents one tissue from one animal used for a single replicate of an experiment. An ANOVA, followed by a Dunnett multiple comparisons test, was used to compare the percentage of change from original weight between treated groups and normals. An ANOVA followed by the Tukey-Kramer multiple comparisons test was used to compare the macroscopic damage score between groups at each time point. To compare the microscopic damage score variables between the groups at different time points and the percentage of animals with dysplasia, the Ganes-Howell multiple comparison procedure was used. A nonparametric Mann-Whitney test was used to compare the correlation of microscopic damage score and dysplasia. Statistical analyses were done using Graph Pad Instat V3.0 (Graph Pad Software) and PEPI V4.0 (J. H. Abramson and Paul Gahlinger, Sagebrush Press, 2001). In all cases, P < 0.05 was considered to represent a significant difference.

**Results**

Throughout the entire study (up to 15 weeks) all animals had normal appearance and behavior. The experimental groups lost weight during the first 3 days of reactivation, probably due to a combination of the i.v. administration of TNBS and perhaps stress from the procedure (Fig. 1B). After this time point, all rats started to gain weight; however, the prolonged reactivation animals continued to weigh significantly less than normal controls until week 15 (114 days) when they were sacrificed (P < 0.05; Fig. 1). There were no significant differences in the weight of the animals from the reactivated DMH or saline-DMH groups at the day of sacrifice compared with normal group (Fig. 1A).

Colonic damage variables, including ulceration, thickness, adhesions, and diarrhea, were measured in all animals, scored, and added to give a total macroscopic score. Although some animals had diarrhea during the study, none of the animals presented with diarrhea at time of sacrifice. The total macroscopic score was ~1 in normal animals at all time points. This is equivalent to the normal thickness (mm) of the bowl wall. It was observed that in both the prolonged reactivation and the reactivated DMH groups, there was an increase in the total macroscopic damage score compared with normal, reaching significance at 10 weeks (P < 0.01). Animals from the saline-DMH group did not have significantly different total macroscopic damage scores from normal (Fig. 2).

When the individual variables were analyzed, there was no difference in the ulceration score between the groups at 5 weeks. At this time, adhesions were present in both the prolonged reactivation and reactivated DMH groups. The thickness of the colon was higher in both the prolonged reactivation and reactivated DMH groups. At 10 weeks, normal rats still did not have any ulceration or adhesions. Ulceration, adhesions, and thickness scores were highest in the reactivated DMH group. In this group, the adhesion score was significantly higher than in normal animals (P < 0.01) or saline-DMH (P < 0.01) at the same time point. At 15 weeks, all animal groups, except for normal, had ulcerations, adhesions, and an increase in colon thickness, with the highest scores again in the reactivated DMH group (data not shown).

![Figure 1. Percentage of weight change in the different groups. Rats were weighed weekly to monitor any weight change in comparison with their original weight. A, the prolonged reactivation group and the saline-DMH showed the least weight gain during the 15-wk treatment (n = 5–17); bars, SE; *, P < 0.05 compared with normal. B, amplification to show the first 20 d of treatment. All of the experimental groups lost weight during the first 3 d of reactivation compared with normal (n = 5–17); bars, SE; *, P < 0.05.](image-url)
Colon length can be a useful indicator of colitis, because the inflamed colon gets shorter and thicker (28). The average colon length in the normal animals was 14.60 ± 0.49 cm at 5 weeks and did not change significantly over time. There was a significant decrease in the average colon length of the animals in the reactivated DMH group at 10 weeks (P < 0.001) compared with normal, indicating overall colon shortening due to the effects of the combination of both the proinflammatory and procarcinogen compounds. In contrast, the saline-DMH group and the prolonged reactivation group were not significantly different from normal controls at any time point, although the average colon length tended to be shorter (data not shown).

There was a significant increase in the total microscopic score in comparison with normal at 5 weeks in all the experimental groups, at 10 weeks in the prolonged reactivation (P < 0.05) and the reactivated DMH group (P < 0.001), and at 15 weeks in the reactivated DMH group (P < 0.05; Table 1). We further compared the total microscopic damage score between the groups at the different time points per segment of the colon. In general the distal colon had the highest microscopic scores, followed by the mid and then proximal (data not shown).

<table>
<thead>
<tr>
<th>Table 1. Average of microscopic variables</th>
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<tr>
<td>Group</td>
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<tr>
<td>5 wk</td>
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<tr>
<td>Normal</td>
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<td>Saline-DMH</td>
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<td>Prolonged</td>
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<td>Reactivated DMH</td>
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<tr>
<td>10 wk</td>
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<tr>
<td>Normal</td>
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<td>Saline-DMH</td>
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<td>Prolonged</td>
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<td>Reactivated DMH</td>
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<tr>
<td>15 wk</td>
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<tr>
<td>Normal</td>
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<tr>
<td>Saline-DMH</td>
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<tr>
<td>Prolonged</td>
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<tr>
<td>Reactivated DMH</td>
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</table>

NOTE: n = 5–6 (±SE).

*P < 0.05, cf. normal within same time point.
† P < 0.01, cf. normal within same time point.
‡ P < 0.05, cf. prolonged within same time point.
†† P < 0.001, cf. normal within same time point.
§ P < 0.05 cf. prolonged 5 wk.
between groups at the same time point, the saline-DMH group had a significant increase in goblet cell depletion (P < 0.05) and crypt abscess (P < 0.01) in comparison with normal at 5 weeks. The reactivated DMH group had a significant increase in goblet cell depletion in comparison with normal (P < 0.01) and prolonged reactivation (P < 0.05) at 10 weeks. Goblet cell depletion and presence of crypt abscesses were rarely seen in normals. There were no significant differences in loss of mucosal architecture, cell infiltration, and muscle thickening between the groups at any time point (Table 1).

Dysplasia was found in all groups except normal (Fig. 3). At 5 weeks, the incidence of dysplasia in any of the colon segments (proximal, mid or distal) was significantly higher in the prolonged reactivation group (P < 0.05) and reactivated DMH (P < 0.01) compared with normal or saline-DMH (P < 0.05). At 10 weeks, the reactivated DMH group showed a significant increase in the incidence of dysplasia in comparison with either normal (P < 0.001), the saline-DMH group (P < 0.01), or the prolonged reactivation group (P < 0.05). At 15 weeks, both the saline-DMH and the reactivated DMH group had a significantly higher incidence of dysplasia compared with normal (P < 0.05). These results suggest that the prolonged reactivation group had a higher incidence of dysplasia during the early stages of the treatment (5 weeks), whereas in the saline-DMH the dysplasia tended to occur later (15 weeks); the presence of dysplasia in the reactivated DMH group was fairly constant at the time points studied (Table 2).

Although normal animals had no dysplasia in any segment of the colon (Fig. 4A), we found some cases of nonspecific inflammation, cryptitis, microabscess, and follicular lymphoid infiltration, which was more evident as the animals got older (15 weeks). The saline-DMH group also presented with signs of inflammation, although they had never been exposed previously to the TNBS. IBD and nonspecific inflammation were the most common pathologic diagnosis in any of the segments of the colon from this group, with dysplasia more evident at 15 weeks. Carcinoma in situ was also found in this group at 5 weeks in the mid colon. The prolonged reactivation group presented inflammatory changes, including IBD, nonspecific inflammation, and lymphoid proliferation in any segment at any of the times studied (Fig. 4B). This group presented with dysplasia and carcinoma in situ as early as 5 weeks, and abnormally large nuclei were routinely observed (Fig. 4C and D). The reactivated DMH animals showed inflammatory changes and transition to dysplasia with a similar incidence at the three time points, but no carcinoma was found in any of the colon segments (Fig. 4E and F).

When the pathologic changes were compared with the microscopic damage scores, we found that the animals with low-grade dysplasia tended to have a higher microscopic damage score in comparison with rats diagnosed with no dysplasia. Specifically, in the mid colon at 5 weeks and in the distal colon at 15 weeks, there was a significant increase in the microscopic damage score in those animals with low-grade dysplasia compared with those that had no dysplasia (P < 0.05; data not shown).

Prescreening for possible mutations in the K-ras gene done by DHPLC limited the amount of samples to be sequenced, and showed no evidence of mutations in any of the samples analyzed. The APC gene, including the three overlapping segments of exon 15, was analyzed for mutations using representative samples of each diagnosis based on the pathologic evaluation. The results of APC segment A showed a thymidine to cytosine base substitution at nucleotide 2,778 in 18 of 29 of the samples sequenced, including tissues pathologically confirmed as normal. However, the substitution of a thymidine for a cytosine produced no change in the amino acid codification. Sequencing analysis of the remaining two segments, APC B and ABC C, showed no evidence of mutation. Sequencing analysis of exons 6 and 7 of the p53 gene showed a nucleotide-based substitution from adenine to cytosine in 22 of 29 samples at nucleotide 1,327. As with the APC gene, this base change was also seen in tissues diagnosed as normal. In this case the base substitution produced a change in the amino acid
Discussion

The risk of colorectal cancer increases with duration of the disease in ulcerative colitis patients, with an estimated risk of 2% at 10 years, 8% at 20 years, and 18% at 30 years (29). DMH has been used widely to produce tumors similar to that found in human sporadic colon cancer (15). In the present study, those animals receiving DMH alone, with no prior inflammation, acted as a control group for the ability to induce carcinogenic changes in our rats. During the development of our model of colitis-associated dysplasia, we did not know whether continuing the inflammation alone would be sufficient to push the pathology toward dysplasia, so we also included a group of animals which received DMH after inflammation, anticipating that the DMH would trigger the neoplastic process and drive the inflammation to dysplasia.

From the groups tested, both the prolonged reactivation and the reactivated DMH seem to be the best options for the development of a new rat model of colitis-associated colon cancer. For future studies, we suggest the use of the prolonged reactivation group, because carcinoma was found in some samples and the treatment protocol resembles more the prolonged chronic inflammation that occurs in humans without requiring additional carcinogen administration. In this study, colon thickness was measured as part of the macroscopic damage score, along with the presence of adhesions, diarrhea, and ulceration. As expected, in chronic colitis, macroscopic scores were not as high as in acute models of colitis (22), because over time, the colon is exposed to healing processes and epithelial renewal mediated by the activation of immune mechanisms. However, an overall increase in the macroscopic damage in both of the groups reactivated with the TNBS was still observed.

Microscopically, we found nonspecific inflammation and active colitis in the saline-DMH group, which is interesting because this group did not receive any previous exposure to TNBS; thus, no colitis was initially induced. This suggests that DMH can cause dysplasia with the development of inflammation as an intermediate stage. Another interesting finding was the higher incidence of dysplasia in both the proximal and distal colon compared with the mid region.

The microscopic analysis also showed that rats from the same groups at the same time points may or may not develop dysplasia. This diversity of diagnosis in the same group of animals and the susceptibility to inflammation or dysplasia could be explained by differences in the host immune system and genotype (30). The most abundant type of dysplasia found in the colonic tissues was low-grade dysplasia, followed by high-grade dysplasia in six tissues and carcinoma in three tissues. To induce a more carcinogenic stage in these animals, we would probably need to increase the length of treatment time, but the present study focused on the transition of colitis to dysplasia and this was clearly shown. We also found regions where there was a transition from healthy cells to inflammation to early dysplasia, suggesting that our model works as a colitis-associated dysplasia model.

The novelty of this model of colitis-associated dysplasia in comparison with other models is that the administration of the compounds more closely mimics what happens in the human condition. Previous studies by other groups have shown first the procarcinogen compound (DMH or azoxymethane) followed by the administration of the inflammatory compound (TNBS or DSS; refs. 31, 32). This treatment combination causes tumors mostly in the mid colon (32). In our study, we induced the colitis intracolonically only once, and thereafter, the TNBS administration was carried out systemically. Although two polyps were found, one in the prolonged reactivation group and the other one in the reactivated DMH group, those polyps were classified as pseudopolyp and benign, respectively.

Although some previous studies have shown that a carcinogen or proinflammatory compound by itself does not cause a tumorigenic effect (33), many others have successfully induced tumorigenesis with DMH or azoxymethane alone (15, 34). Recognizing the need for models to study the dysplasia-cancer sequence, a few more recent studies have investigated the use of the proinflammatory agent DSS, most notably those by Cooper et al. (3, 35). The present study now shows that prolonged reactivation with another proinflammatory compound, TNBS, also results in a shift from colitis to dysplasia and carcinoma. The incidence of dysplasia in our studies was somewhat higher and at earlier time points than those with DSS, which ranged from <10% to almost 40%, depending on the number of cycles given, percentage of DSS, and time of sacrifice (3, 36). In addition, the majority of the DSS studies have been undertaken in mice, whereas the current model offers a novel alternative rat model, which seems to be quicker in onset with zero mortality. Taken together, these studies suggest that a long-term colitis model is a feasible option to study colitis-associated colon cancer.

### Table 2. Percentage of animals with dysplasia or carcinoma

<table>
<thead>
<tr>
<th>Group</th>
<th>LGD</th>
<th>HGD</th>
<th>C</th>
<th>LGD</th>
<th>HGD</th>
<th>C</th>
<th>LGD</th>
<th>HGD</th>
<th>C</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline-DMH</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prolonged</td>
<td>60</td>
<td>40</td>
<td>40</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Reactivated DMH</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>83</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Percentage of animals per treatment group with a diagnosis of low-grade dysplasia, high-grade dysplasia, or carcinoma at each time point. Abbreviations: LGD, low-grade dysplasia; HGD, high-grade dysplasia; C, carcinoma.
Some previous studies have shown a specific sequence of mutations in K-ras, APC, and p53 genes in sporadic colon cancer (35, 37, 38); however, others have suggested that there is a variation in the arrangement of the mutations between the genes in colitis-associated colon cancer (39, 40). In our study, a representative selection based on the pathologic diagnosis rather than by group or time point was used to correlate genetic changes with the stage of transition. The mutation analysis done in this study showed that there was no correlation between the samples diagnosed with dysplasia or carcinoma and the molecular alterations found in the K-ras, APC, and p53 genes. We observed a base substitution at nucleotide 2,778 of the APC gene and at nucleotide 1,327 of exon 6 of the p53 gene. Eleven samples harbored both mutations. These results might represent single-nucleotide polymorphisms or infer that the changes are not associated with the treatment.

The fact that most of the active IBD, dysplasia, and carcinoma diagnosed were at focal sites in the tissue, not the tissue as a whole, may also have reduced the probability of finding mutations in the genes studied. Another factor accounting for the low incidence of mutations in our samples may be our concentration on the transition stage from inflammation to dysplasia and not the later stages with tumor or malignant polyps. If there are mutations, they might be too few to be detected. Previous studies in genetic mutation analysis have shown that none of the current methods of analysis used in this study, either DHPLC or direct sequencing, has a 100% rate of detection, and suggest a combination of both methods to improve the efficiency in the molecular diagnosis (41). K-ras, APC, and p53 are genes that have been highly related to colon cancer in both humans and rodents; nevertheless, the molecular changes of these genes associated with sporadic or colitis-associated colon cancer are still very variable according to the literature (42).

In conclusion, the few gene alterations found in this study do not disqualify this model as a useful tool to study molecular markers for the detection or prevention of early stages of colon cancer. Upon further validation, these and other markers that are identified using this animal model may be useful in patients with IBD to screen their susceptibility for the development of colon cancer. This new model will allow a more in-depth analysis of the transition from chronic inflammation to dysplasia and may help shed light on why some patients maintain active IBD whereas others develop colon cancer. It will be important to expand these studies into investigations of the molecular pathways associated with these three important cancer genes, as well as other pathways.

![Figure 4. Microscopic results from colonic tissue stained with H&E. A, mid colon of a rat from the untreated group showing normal appearance of the mucosa. The crypts conserved their normal architecture, and there is no presence of neutrophils or inflammatory cells (200×). B, mid colon of a prolonged reactivation rat showing the presence of two large lymphoid follicles invading the lamina propria, causing loss of mucosal architecture (400×). C, high-grade dysplasia in the proximal colon of a prolonged reactivation rat showing the presence of crypt abscesses (arrow) and very large nuclei (200×). D, presence of atypical mitosis (arrow) in a dysplastic gland of a prolonged reactivation rat (oval showing normal mitosis). E, tissue from the proximal colon of a reactivated DMH rat showing the transition from a normal appearance to inflammation to low-grade dysplasia; oval, showing microabscess; square, showing crypt abscess; arrow, showing low grade dysplasia (200×). F, low-grade dysplasia in the proximal colon of a reactivated DMH rat. Arrows, the region with low-grade dysplasia in the upper one-third of the colon mucosa (200×).](image-url)
of cytokine activation, such as the epidermal growth factor receptor (43, 44) and Cox-2 (45), which have been previously studied in colitis models.

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