Susceptibility of Msh2-deficient Mice to Inflammation-associated Colorectal Tumors

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INTRODUCTION

Colorectal cancer is one of the most serious and frequent complications of long-standing IBD, particularly in chronic ulcerative colitis, which 10 years after onset, the cumulative risk is 0.5–1% per year in pancolitis patients (1). There are significant differences in the early pathogenesis and molecular alterations of ulcerative colitis-associated tumors as compared with common sporadic colorectal cancer implying that at least the early carcinogenesis is different (2). Dysplasia in sporadic cancers usually presents as a well-defined polypl, whereas dysplasia in ulcerative colitis is diffuse and macroscopically flat. It is well known that failure of cellular mechanisms to detect and repair defects in the DNA sequence is associated with colon carcinogenesis (3, 4). MSI as a marker of DNA mismatch repair deficiency has been described in hereditary and common colorectal cancer, and a standard panel of markers is used to grade tumors to MSI-H, MSI-L, and MSS (5). However, the frequency, degree, and significance of MSI in IBD-associated tumors is unclear. In one study, 9% of 148 colitis tumors were MSI-H, and 11% were MSI-L, with loss of DNA mismatch repair gene MLH1 expression in half of the MSI-H specimens (6). This frequency distribution resembles that of MSI found in common colorectal cancer (5), whereas another study describes a lower percentage of MSI-H tumors with loss of expression of MSH2 but normal MLH1 expression (7). Although the different studies display divergent results, they imply acquired mismatch repair deficiency in a small proportion of tumors triggered by chronic inflammation.

Whereas the pathogenesis of IBD cancer remains poorly understood, there is little dispute that the immune system plays a central role in the initiation and regulation of the tissue injury caused by free radical species (8) and metalloproteases (9) characteristic of the disease. Experimental animal models of IBD have provided new insights to different aspects of the disease in humans (10). Colitis can be induced in mice or rats by supplementing the drinking water with DSS (11, 12). This model shows evidence of free radical species, oxidative injury, and depletion of antioxidants in the inflamed mucosa (13–15) similar to that in human IBD (16, 17). Initially, DSS produces an acute colitis, which becomes chronic after repeated cycles of DSS/water. DSS is not a carcinogen on its own, but dysplasia and adenocarcinoma develop in a small subset of mice with chronic colitis (18).

The Msh2-deficient mice have a defect in one of the DNA mismatch repair genes that in humans cause susceptibility to HNPCC (19–21). However, there are some important differences. Mice homozygous for the Msh2 defect (Msh2-/-) develop late-onset adenocarcinoma in the small intestine rather than in the colon, the latter of which is the main site of cancer in HNPCC. Mice heterozygous for the Msh2 defect do not develop spontaneous small intestinal or colon tumors in contrast to heterozygous carriers of the corresponding human mutations. The aim of this study was to examine the effect of chronic inflammation on the frequency of colorectal dysplasia and cancer in Msh2-deficient mice and examine the MSI status of the tumors. We show that Msh2-/- mice develop a significantly higher frequency of colon tumors than the heterozygous Msh2+/− and wild-type Msh2+/- mice. The MSI status of the lesions was also different, with the Msh2-/- mice developing tumors with MSI, whereas the heterozygous and wild-type mice developed mostly MSS neoplasia.

This system offers opportunities to characterize the different molecular pathways of carcinogenesis operating in chronic colitis.
and MSS specimens to confirm MSI status. Primer sequences for all dinucleotide markers showed instability or MSI-L if only one marker was unstable. An extra peak than at least one of the neighboring fragments in tumor tissue, this was classified as MSI. In addition, extra peaks often appeared that were negligible or not present in normal tissue.

Analysis of Short Mononucleotide Repeats. Three short mononucleotide repeats, JH102, JH104 (24), and a A 4 GA 4 repeat from the TGFβRII (25), were analyzed for insertion-deletion mutations in the dysplasia and cancer specimens. The primer sequences for TGFβRII were 5'-CAT TAC TCT GGA GAC GTT C (forward strand) and 5'-TCG TTG CAT CTC TCC ATG TTA C (reverse strand). PCR was carried out with fluorescent primers as above, except that the annealing temperature was 56°C with a 7-min final extension. Fragments were visualized using the ABI 310 Genetic Analyzer. The JH104 and the TGFβRII fragments were also sequenced to confirm results obtained from PCR fragment length analysis.

Statistical Analysis. The effect of the Msh2 genotype on the frequency of dysplasia and carcinoma was analyzed by χ² with exact probability (program StatXact; Cytel Corp., Cambridge, MA). The frequencies were subdivided into four categories (normal, low-grade dysplasia, high-grade dysplasia, and adenocarcinoma), and pair-wise comparisons were made between the different genotypes. Daily basal water consumption (mean/mouse) was determined and analyzed during the first two cycles of DSS supplementation by general linear model ANOVA with genotype and sex as categorical variables (program SYSTAT 7.0.1; SPSS, Inc., Chicago, IL).

RESULTS

Induction of Chronic Colitis. Groups of Msh2+/+, Msh2−/−, and Msh2+−/− mice were given repeated cycles of DSS/water, which caused chronic diarrhea and occasional rectal bleeding. The mean daily consumption of DSS-supplemented water did not differ significantly between genotypes (P = 0.39, general linear model ANOVA), but males consumed more than females (P < 0.001) as expected according to their higher body weight. This was compensated by giving the females 1–2 extra days of DSS in the first two cycles. The least-square means were 3.85, 3.78, and 4 ml/day for the three genotypes +/+, +/−, and −/−, respectively. Nine DSS-treated Msh2−/− mice were sacrificed after three cycles of DSS, because of severe symptoms of colitis or bowel obstruction (7 mice) or large thymic lymphoma (2 mice). Eight of 9 Msh2−/− mice had developed dysplasia or cancer (4 low-grade, 3 high-grade dysplasias, and 1 adenocarcinoma). Six Msh2+/+ and 2 Msh2+−/− mice also had to be sacrificed at this time point because of severe colitis symptoms, but dysplasia had developed in only 2 Msh2+/+ mice (one low grade and one high grade) and 1 Msh2+−/− mouse (high grade). The rest of the mice were sacrificed after five or eight cycles of DSS.

Microscopically, mice treated with DSS, showed diffuse and often transmural chronic active inflammation of the colon (Fig. 1B). The inflammatory changes were more marked distally. The colitis was characterized by crypt distortion and crypt loss, basal plasmacytosis, cryptitis, focal crypt abscess formation, accumulation of foamy macrophages, and lymphoid aggregates. There was often squamous metaplasia of the rectum. Paneth cell metaplasia was not a feature of the inflammatory changes. There was no significant small bowel inflammation. The severity of inflammation was similar in all three genotypes with ~80–90% of DSS-treated mice showing symptoms of severe chronic inflammation.
Colorectal Dysplasia and Cancer. Most (28 of 30) Msh2+/− mice developed some degree of dysplasia or cancer. Almost half (46.7%) of the Msh2+/− mice developed high-grade dysplasia (Table 1 and Fig. 1C), whereas only 12.5% of the Msh2+/− (P = 0.002, χ²) and 8% of the Msh2+/− mice had this lesion (P = 0.0001). After eight cycles, the proportion of adenocarcinoma increased in both the Msh2+/+ and Msh+/− mice, but the overall frequency of neoplasia was still 35 and 46%, respectively. About 81% of adenocarcinomas showed mucinous differentiation (Fig. 1D), which is also a feature of human ulcerative colitis. Adenocarcinoma was associated with low- or high-grade dysplasia in 6 of 12 Msh2+/+ mice, 9 of 13 Msh2+/− mice, and 3 of 4 Msh2+/− mice. Table 1 summarizes the pathology results for each

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No treatment</th>
<th>DSS</th>
<th>DSS</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neoplasia</td>
<td>Indef + LGD b</td>
<td>HGD</td>
<td>AdCa</td>
</tr>
<tr>
<td>3–5 cycles of DSS a</td>
<td>25.0 (6/24)</td>
<td>12.5 (3/24)</td>
<td>16.7 (4/24)</td>
<td></td>
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<tr>
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<td>24.0 (6/25)</td>
<td>8.0 (2/25)</td>
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<tr>
<td>Msh2+/−</td>
<td>0 (0/10)</td>
<td>33.3 (10/30)</td>
<td>46.7 (14/30)</td>
<td>13.3 (4/30)</td>
</tr>
<tr>
<td>8 cycles of DSS c</td>
<td>8.1 (3/37)</td>
<td>5.4 (2/37)</td>
<td>21.6 (8/37)</td>
<td></td>
</tr>
<tr>
<td>Msh2+/+</td>
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<td>15.2 (7/46)</td>
<td>6.5 (3/46)</td>
<td></td>
</tr>
<tr>
<td>Msh2+/−</td>
<td>0 (0/29)</td>
<td>15.2 (7/46)</td>
<td>6.5 (3/46)</td>
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</table>

a Effect of the Msh2+/− genotype on dysplasia/carcinoma development is statistically significant after three to five cycles of DSS/water (Msh2+/− vs. Msh2+/+, P = 0.002, χ²; Msh2+/− vs. Msh2+/−, P = 0.0001; Msh2+/+ vs. Msh2+/−, P = 0.69).

b Indef, indefinite; LGD, low grade; HGD, high-grade dysplasia; AdCa, adenocarcinoma. The frequency of indefinite and low-grade dysplasia is combined because of the occasional difficulty in differentiating between the two (22).

c P = 0.77.
group of mice. In the case of multiple lesions found, the histologically most advanced tumor was considered for each mouse.

**Microsatellite Analysis.** Lymphomas and intestinal tumors from the Msh2+/− mice are known to display MSI (19–21, 26). We first tested 20 of these spontaneous tumors from the Msh2+/− mice with markers D15Mit93, D17Mit72, uPAR, and pro-1 and observed MSI in all specimens with one or more of the markers. Colorectal dysplasias and carcinomas from 83 DSS-treated mice were then analyzed for MSI (Table 2). Both the inflammation-associated colorectal tumors and the spontaneous intestinal adenocarcinomas from the Msh2+/− mice displayed subtle changes from the reference microsatellite patterns (Fig. 2, A and B). The most sensitive indicator of MSI was the T24 tract in an intron of the uPAR gene. Altogether, 21 of 27 Msh2+/− colorectal tumors showed instability in this locus and in at least one other marker (Fig. 2B). Therefore, these tumors were classified MSI-H following the standard criteria for human colorectal cancer (5). Three of the 27 Msh2+/− colorectal tumors showed instability in only one marker (MSI-L), and three were MSS. Other areas of the intestinal tract, such as stomach and proximal small intestine in the DSS-treated mice, showed the same pattern as the normal colon in untreated Msh2+/− mice. The Msh2+/− tumors analyzed included three adenocarcinomas and 14 high-grade, 8 low-grade, and 2 indefinite dysplasias (Table 2). Of these, 1 high-grade and 2 low-grade dysplasias were MSI-L, and 2 low-grade and 1 indefinite dysplasia were MSS. There was no correlation between the MSI status and the location of the tumor in the colon.

Tumors from 3 Msh2+/+ and 4 Msh2+/− mice were MSI-L, and none showed instability in the uPAR marker. All MSI-L and MSS tumors were also analyzed with a further two dinucleotide markers, D17Mit123 and D17Mit185, but these results did not change the MSI status of any sample. The Msh2+/− tumors that were MSI-L were also studied for loss of heterozygosity using the Msh2 locus-specific PCR, but both the wild-type (Msh2+/−) and the targeted (Msh2−/−) alleles were retained in these specimens (data not shown).

Regions of colon with no identifiable dysplasia or cancer were selected for additional MSI analysis from 22 DSS-treated Msh2+/− mice that had developed tumors. All tissue sections surrounding the identified tumor in the block were analyzed and included both inflamed and normal tissue. MSI was evident in 15 of 22 specimens, particularly with the mononucleotide markers (Fig. 2C). This implies that some MSI is induced by colon inflammation and can precede dysplasia in Msh2+/− mice. The exceptions included 2 mice whose tumors were MSS and 5 mice whose tumors were MSI-H.

**Mutations in Short Mononucleotide Repeats and the TGFβRII Gene.** To examine whether the MSI observed would also cause deletion-insertion mutation in shorter repeat sequences, an additional three genes were analyzed. The JH102 and JH104 repeats have been reported previously to be good indicators of MSI in tumors of Msh1-deficient mice (24). JH102 is an A6 repeat in the coding region of the mouse Apc gene. JH104 is located in an intron of the Msh3 DNA mismatch repair gene. No insertion-deletion mutations were found in any of these markers in 10 MSI-H Msh2+/− colorectal tumors analyzed. The JH104 marker was also found to be polymorphic, with a T9 repeat in the C57BL/6J strain and a T10 repeat in the 129/OLA strain. The A6GA4 repeat in the coding region of the TGFβRII gene corresponds to the A10 repeat known to be mutated in human MSI-H colorectal cancers (27) but has not been studied previously in intestinal or colorectal tumors in these mice. We could not find any evidence of insertion-deletion mutations of the A6GA4 repeat in colorectal tumor specimens from 21 DSS-treated Msh2+/− mice.

**DISCUSSION**

The recognition of DNA mismatch repair genes in hereditary and common colorectal cancer (3, 4) has led to the analysis of the role of these genes in IBD-associated cancer (6, 7). Genetic association studies implicated mismatch repair genes as minor susceptibility genes when the frequencies of an MSH2 splice site substitution were compared in two groups of patients with ulcerative colitis, one with high-grade dysplasia and cancer and one without tumors (28). It was noted that this substitution was more common in the patient group with tumors and concluded that a variant of MSH2, which is not normally deleterious, may predispose to cancer in the setting of chronic colitis. A later study (29) was unable to confirm the association of colitis cancer with MSH2. A significant association between Crohn’s disease/ulcerative colitis, certain polymorphic alleles of the MLH1 gene, and a microsatellite marker located in an intron of MLH1 has been reported (30). However, there is no association between HNPCC and an increased risk for IBD.

This study describes the effect of a DNA mismatch repair defect on the development of dysplasia and cancer when triggered by chronic inflammation. We analyzed mice that were either homozygous, heterozygous, or wild type for the Msh2 DNA mismatch repair defect.

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**Table 2** Percentage of mice showing MSI in colorectal dysplasia and cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Indef*</th>
<th>LGD</th>
<th>HGD</th>
<th>AdCa</th>
<th>Total no. of mice</th>
<th>MSI-H % (n)</th>
<th>MSI-L % (n)</th>
<th>MSS % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msh2+/+</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>26</td>
<td>0 (0/26)</td>
<td>11.5 (3/26)</td>
<td>88.5 (23/26)</td>
</tr>
<tr>
<td>Msh2+/−</td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>13</td>
<td>30</td>
<td>0 (0/30)</td>
<td>13.3 (4/30)</td>
<td>86.7 (26/30)</td>
</tr>
<tr>
<td>Msh2−/−</td>
<td>2</td>
<td>8</td>
<td>14</td>
<td>3</td>
<td>27</td>
<td>77.8 (21/27)</td>
<td>11.1 (3/27)</td>
<td>11.1 (3/27)</td>
</tr>
</tbody>
</table>

* Indef, indefinite; LGD, low grade; HGD, high-grade dysplasia; AdCa, adenocarcinoma.
There was no difference in the severity of inflammation between the three genotypes. Heterozygosity for the Msh2 defect did not increase tumor susceptibility in the setting of chronic inflammation compared with wild type. Heterozygous carriers of a MSH2 gene defect in humans ultimately develop HNPCC from their late teens as the wild-type allele is mutated in colon epithelial cells. It is not known what triggers the somatic mutation in HNPCC, and no data are available from human IBD patients who also are MSH2 mutation carriers. The majority of the Msh2+/− and wild-type tumors in our study appeared to develop independently of the mismatch repair system as very little MSI was found.

In contrast to the maximum frequency of ∼30% of high-grade dysplasia or adenocarcinoma found in the wild-type or Msh2−/− mice, 60% of the DSS-treated Msh2−/− mice developed these tumors. The largest difference was observed in the frequency of high-grade dysplasia, with 12.5, 8, and 46.7% in the Msh2+/−, Msh2−/−, and Msh2−/− mice, respectively, whereas the frequency of adenocarcinoma was similar for each genotype. These results suggest an important connection between the DNA mismatch repair proteins and development of dysplasia and contrast a widely held view that mismatch repair defects accelerate carcinogenesis (31, 32). There is increasing evidence that the mismatch repair machinery is not only activated by mismatched nucleotides but also by DNA damaging agents (33, 34). It has been suggested that the mismatch repair proteins may signal damage-induced apoptosis through a direct-sensor signaling process (32, 35). Our study suggests that inflammation-induced damage may also be controlled by mismatch repair proteins, such as MSH2. It will be important to determine whether chronic colitis involves a similar apoptotic pathway (32, 33).

We also observed a significant difference in the frequency of MSI, with the majority of Msh2−/− tumors being MSI-H. However, the individual MSI patterns were much more subtle than we have observed in human colorectal cancer specimens using similar methods but no different from the spontaneous intestinal cancers from the same mice. Nevertheless, the tumor MSI patterns were absolutely reproducible and quantifiable. Interestingly, we were not able to detect any instability in short mononucleotide repeats, which have been reported previously to be mutated in tumors from mismatch-deficient mice. These included the A6GA4 repeat in the coding region of the TGFBR1 gene, as reported to be mutated in Msh2−/− lymphomas (25). The corresponding A10 repeat is also an important mutation target in human MSI-H colorectal cancers (27). This indicates that other target genes may be mutated or abnormally regulated in this model.

In the majority of DSS-treated Msh2−/− mice tested, MSI was also detected in the colon tissue surrounding the tumor, implying that some MSI is already induced by inflammation and precedes dysplasia. Instability at one or more microsatellite loci has also been found in a proportion of human inflamed non-neoplastic lesions in IBD patients (28, 29, 36). It has been suggested that this is caused by saturation of normal DNA repair mechanisms in inflamed colon epithelium because of markedly increased cell turnover and proliferation in IBD (29). Our results are compatible with the hypothesis that the increased proliferation induces widespread DNA replication errors, which are repaired normally until the DNA repair systems become saturated. As a result of defective mismatch repair already present in the Msh2+/− mice, this is quickly manifested by the appearance of MSI in the inflamed colon tissue. In the wild-type and Msh2−/− mice, chronic inflammation causes the same DNA damage, but the intact mismatch repair system is able to repair more of the damage.

This still leaves a high proportion of MSS dysplasia and cancer that cannot be explained on the basis of temporary saturation of the DNA mismatch repair system and probably includes the majority of human IBD cancers. Therefore, other mechanisms will need to be looked at, such as gene silencing by hypermethylation of CpG-rich promoters and injury to DNA bases directly as a result of the inflammatory response. In conclusion, we have described a novel application of a previously well-characterized animal model of chronic colitis. We have shown that by studying colorectal dysplasia and cancers developing in mice that are either homozygous, heterozygous, or wild type for a DNA mismatch repair defect, parallels can be drawn to human chronic colitis-associated cancers. Additional molecular genetic analysis of this model is likely to reveal more insights to the early pathogenesis of dysplasia and the different molecular mechanisms of carcinogenesis operating in IBD cancers.

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