Enzyme-altered Foci in Colons of Carcinogen-treated Rats

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ABSTRACT

The distal colon and rectum from male F344 rats treated with 15 mg/kg 1,2-dimethylhydrazine 2HCl (DMH) for 20 weeks were analyzed for focal areas of enzyme alteration. Tissues were embedded in methacrylate at 4°C and cut in 2- to 4-μm serial sections. In DMH-treated rats, 8.8 ± 2.4 feci/cm² of examined mucosa were observed at 20 weeks and 7.7 ± 1.1 feci/cm² at 31 to 52 weeks, compared with 1.2 ± 0.6 feci/cm² in control rats (P = 0.01). The number of feci at 31 to 52 weeks compared with 20 weeks did not change significantly, but the area of altered rectal mucosa increased from 0.22 ± 0.2% at 20 weeks to 1.47 ± 0.6% at 31 to 52 weeks (P = 0.051). Most foci had decreased N-acetyl-β-D-glucosaminidase, α-naphthyl butyrate esterase, and muco in epithelial cells and increased γ-glutamyl transpeptidase in the stroma. Morphologically, the foci varied from normal to overtly dysplastic. Grossly, tumors were identified in 5 of 20 DMH-treated rats killed at 31 to 52 weeks but not in 12 DMH-treated rats killed at 20 weeks or 30 control rats killed at 20 to 52 weeks. These data suggest but do not establish that enzyme-altered foci are putative preneoplastic lesions in the colon.

INTRODUCTION

The development of colon cancer, like most cancers, involves multiple steps over a long period of time and is thought to include "the process of tumor progression, i.e., the conversion of benign tumors to malignant tumors" (1). Although most human colon cancers are thought to originate in adenomas (2, 3), some suggest that the more aggressive cancers develop in small flat adenomas that frequently show severe dysplasia and are difficult to recognize endoscopically (3). The characterization of early precursor lesions of colon cancer may increase our understanding of oncogenesis in this organ and provide additional criteria with which to evaluate the risks of individual patients in developing colon cancer. "Enzyme-altered foci" have been characterized extensively in the livers of rodents treated with carcinogens (4–10). These focal areas exhibit a large number of phenotypic alterations, are thought to be the progeny of single altered cells, and are generally considered to be putative preneoplastic lesions (reviewed by Peraino et al.; Ref. 11). Analogous enzyme-altered lesions reported by Mayer et al. (12) in the colons of rats treated with DMH exhibited obvious architectural abnormalities. Our study was undertaken to investigate the possible existence of enzyme-altered areas in the colons of carcinogen-treated animals that could be detected before structural abnormalities in the crypts became evident. The use of 2- to 4-μm serial sections of methacrylate-embedded tissues allowed multiple enzyme activities to be demonstrated in the same lesions histochemically even when these lesions were very small and, in some cases, without the development of histological and cytological abnormalities that could be detected with conventional hematoxylin and eosin stains (13), the existence of enzyme-altered foci might facilitate the study of colon carcinogenesis by providing possible markers for putative preneoplastic cells at what appears to be one of the earliest stages identified to date.

MATERIALS AND METHODS

Male F344 rats were bred in our animal facility from breeding stock obtained from the NIH Genetics Colony in 1986. The animals were housed in plastic cages with sawdust bedding at a maximum of 3/cage, with food and drinking water ad libitum. They were maintained in a temperature-controlled room (22°C to 24°C) with a 12-h light cycle and weighed weekly. At 6 to 8 weeks of age, control and experimental rats were placed on a purified diet formulated by the American Institute of Nutrition (14) (AIN No. 76A; Teklad, Madison, WI), given their first injection, and maintained on this diet until sacrifice.

Experimental rats received 20 weekly s.c. injections of 15 mg/kg body weight DMH (Aldrich Chemical Company, Milwaukee, WI), dissolved in 0.9% NaCl solution (saline) and neutralized to pH 6.4. This is equivalent to 6.8 mg/kg of the base, dimethylhydrazine. Control rats received 20 weekly s.c. injections of 0.5 ml saline. Rats were given injections of carcinogen in a total exhaust BioChemGard hood (Baker Company, Sanford, ME) and kept in this hood until 1 week after their last injection. Rats were transferred to clean cages before removal from this hood.

Groups of experimental and control rats were sacrificed 20, 31, and 52 weeks from the start of injections, i.e., 1, 12, and 32 weeks after the last injection. All groups contained 10 rats except the experimental group sacrificed at 20 weeks, which contained 12 rats. The animals were anesthetized with ether and killed by exsanguination. The entire colon was removed, cooled in Joklik's modification of minimum essential medium (Hazleton, Lenexa, KS) at 4°C, opened longitudinally, and washed free of fecal matter. The mucosa is defined histologically. The most distal 2 cm of colon was designated as rectum; the 14- to 16-cm segment between the cecum and rectum was divided into three equal portions designated proximal, mid, and distal colon, since the rat does not have distinct segments like the human (15). Grossly identifiable lesions were measured, described, and located in the colon, embedded in methacrylate, and evaluated with HEA-stained sections.

Histological Preparation. The segments of colon were processed as described previously (16) into “Swiss rolls” (17), snap frozen in the vapor phase over liquid nitrogen, sliced at 1- to 2-mm intervals with a razor blade, and thawed with gentle agitation in fixative at 4°C. The tissue was fixed for 2 h at 4°C in 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and embedded at 4°C in glycol methacrylate (JB-4 embedding kit; Polysciences, Warrington, PA), as described by Beckstead and Bainton (18) and Beckstead et al. (19) and used by our laboratory with slight modifications (9, 16, 20–24). The tissue molds and block holders were obtained from Bio-Rad Microscience Division (Cambridge, MA). The methacrylate blocks were stored in a dessicator at 4°C and brought to room temperature prior to sectioning on a Lipshaw 45 Rotary Microtome (Lipshaw Mfg; Detroit, MI) with a glass knife adapter (LKB, Rockville, MD). Most serial sections of tissue were cut 2-μm in thickness, but duplicate 4-μm sections were used to assess acid phosphatase, alkaline phosphatase, and 5' nucleotidase activities.

Tissue Staining. For morphological evaluation, sections were stained sequentially in Harris-modified hematoxylin (Fisher Scientific, Pittsburgh, PA) for 1.5 min, 0.5% aqueous eosin Y for 20 to 60 s, and 0.1% aqueous azure II for 1.5 min (HEA), with washes in distilled water between stains. Substrates and reagents for enzyme and mucin histochemistry were obtained from Sigma (St. Louis, MO) unless otherwise specified otherwise. The histochemical procedures described by Beckstead et al. were

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3 The abbreviations used are: DMH, 1,2-dimethylhydrazine 2HCl; HEA, hematoxylin, eosin, azur; ANBE, α-naphthyl butyrate esterase; GGT, γ-glutamyl transpeptidase; PAS, periodic acid Schiff.

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GGT was demonstrated by the method of Rutenburg et al. (25), as demonstrated of acid phosphatase, alkaline phosphatase, and ANBE (19) as modified slightly by us previously (9, 20, 23) were used for the with the method of Wachstein and Meisel (30) as used previously (9), previously for use with methacrylate sections (23); mucin, with the PAS method of McManus as presented by the Armed Forces Institute of Pathology (28) and adapted to methacrylate (29); and 5'-nucleotidase, with the method of Wachstein and Meisel (30) as used previously (9), with an incubation of 3 h in the presence of L-p-bromotetramisole oxalate to inhibit nonspecific alkaline phosphatase (31).

All of the above phenotypic markers except hexosaminidase previously had been demonstrated to be altered in liver carcinogenesis (9, 11), and all are readily identified in methacrylate-embedded tissues (9, 23). Since liver and colon are both endodermally derived and malignant cells often manifest fetal phenotypes, it seemed likely that these two organs would share some similar phenotypes during carcinogenesis. In addition, Mayer et al. (12) had demonstrated changes in the expression of acid phosphatase in the colons of carcinogen-treated rats, and many (12, 17, 32–34) have described changes in the chemistry or histochemistry of mucin. The metabolism of carbohydrates and glycoproteins is frequent during carcinogenesis (13, 35). Hexosaminidase activity has been altered in both human (36) and rat (35) colonic tumors; it has been shown to be altered in aberrant colonic crypts of carcinogen-treated rats (37). Alkaline phosphatase (38) and GGT (39) are expressed in fetal colon, alkaline phosphatase is expressed in some cell lines derived from colonic carcinomas (40), and GGT was elevated in rat colonic carcinomas induced with DMH (39). 5'-Nucleotidase is altered in many cancers including prostatic carcinoma (41).

Analysis. Up to eight Swiss rolls per rat from the distal colon and rectum were embedded in methacrylate and cut in serial sections. Duplicate slides were processed for each of the histochemically demonstrable enzyme activities and mucin described. This resulted in over 1000 slides that were analyzed microscopically with a 10-power objective for the presence of enzyme-altered foci. A 15-power eye piece fitted with a Whipple-Hausser ocular grid (No. 6585-H10; Arthur H. Thomas, Philadelphia, PA), calibrated with a stage micrometer, was used to measure the sizes of the altered areas. The amount of mucosa analyzed for the presence of foci was quantified with the Bioquant II Image Analysis system (R & M Biometrics, Nashville, TN) by tracing the perimeter of the mucosa on an HEA-stained serial section from each block with a HIPAD Digitizer (Houston Instruments, Austin, TX). The number of altered foci per area of mucosa and the percentage of mucosa altered were computed for each rat whose tissues were embedded and evaluated. Values for the mean and SE for these parameters are reported. Differences between group (unpaired) means were tested by the conventional Student's t test.

RESULTS

In early experiments Swiss rolls from representative areas of the entire length of colon from two DMH-treated rats were evaluated microscopically for histochemically demonstrable areas of altered activity. No areas of altered activity were detected in the cecum, proximal colon, or mid colon, but focal areas of altered activity were observed in the distal colon and rectum with the histochemical phenotypic markers that we used for these two rats. Tissues from the distal colon and rectum from seven experimental and seven control rats, killed 20 weeks after the first injection (1 week after the last injection), were embedded in methacrylate and analyzed microscopically. Small areas of enzyme-altered activity (Figs. 1, 2, and 3) were identified in the distal colon or rectum of all seven experimental rats but in only three of the seven control rats. Crypts adjacent to lymphoid aggregates in both control and experimental rats frequently demonstrated changes that included an altered morphology, a lack of goblet cells especially in the part of the crypt adjacent to the lymphoid tissue, and altered enzymatic activities. Morphological alterations have been noted previously in these glands (42). As in these earlier studies (42), these glands were not included in our assessment of enzyme-altered foci, since their number depended on the amount of lymphoid tissue in the respective sections.

Altered foci generally displayed multiple phenotypic alterations in both the epithelial cells and the stromal cells, but no single marker identified all of the foci observed (Table 1). In the epithelial cells, a decrease in or total depletion of the histochemically demonstrable hexosaminidase activity (Figs. 1, 2a, and 4b) was the most frequent (Table 1) and useful marker for the identification of enzyme-altered foci. Decreased expression of ANBE (Fig. 3) was observed in many of these same cells examined in 2- to 4-μm serial sections. Many enzyme-altered foci had a marked loss of mucin-producing cells (Fig. 2) that was clearly demonstrated with the PAS reaction. Other foci had goblet cells that were markedly decreased in size or goblet cells with much smaller mucin-containing vacuoles (Fig. 1).

The stroma within altered foci frequently had an increase in histochemically demonstrable GGT activity (Fig. 2b). Increased alkaline phosphatase activity was frequently observed in the same stromal areas of the foci; this appeared to be the result of an increase in stromal cells and blood vessels with histochemically demonstrable alkaline phosphatase activity. 5'-Nucleotidase activity was often increased in the stroma within a focus, but some foci demonstrated decreased 5'-nucleotidase activity (Fig. 2c) in what have been described as “pericryptal fibroblast sheath cells” (16, 43, 44). Some altered foci demonstrated both an increase in histochemically demonstrable acid phosphatase activity in the stromal cells around the altered crypts and a decrease in or total depletion of the acid phosphatase activity in the epithelial cells of the focus. Morphologically, the altered foci varied from normal (Fig. 3) to overtly dysplastic (Fig. 4). When there were histological or cytological alterations in foci, epithelial cell changes included loss of nuclear polarity (Fig. 4), increase in nuclear size (Figs. 2 and 4), decrease in number and size of mucin-producing cells (Figs. 1, 2, and 4), and hyperplasia of the columnar cells. The proportion of stromal elements was often increased (Fig. 2).

The average size of a focus in histological preparations was 0.07 ± 0.05 mm² (range, 0.01 to 0.23 mm²) at 20 weeks and 0.13 ± 0.08 mm² (range, 0.05 to 0.30 mm²) at 31–52 weeks.
Fig. 2. Serial sections of an enzyme-altered focus consisting of a single gland (arrow) with an increased amount of stroma that demonstrates decreased epithelial hexosaminidase (a), increased GGT both in the stroma and on the luminal surface of the altered gland (b), and 5'-nucleotidase activity (c); methyl green counterstain. Note the lack of pericryptal fibroblast sheath cells with 5'-nucleotidase activity as contrasted with the adjacent normal glands (arrowheads). Bar = 30 µm.

Fig. 3. An enzyme-altered focus that appears morphologically normal in this slide and in a serial section stained with HEA. ANBE activity is demonstrated by the red-brown reaction product that appears black in the photomicrograph; methyl green counterstain. Bar = 50 µm.

The mean frequency of altered foci was much greater (P = 0.010 for the combined regions) in tissues from the seven experimental rats that received DMH than that observed in the control rats (Table 2). Evaluation of the distal colon and rectum from two rats killed 31 weeks and from four rats killed 52 weeks after the first injection demonstrated the persistence of enzyme-altered foci for 12 to 33 weeks after the end of carcinogen treatment in all (six) of the rats from which tissues were analyzed microscopically (Table 3). The mean number of foci at 31 to 52 weeks (7.7 ± 1.1 foci/cm²) compared with 20 weeks (8.8 ± 2.4 foci/cm²) did not change significantly, but there was a marked increase (P = 0.051) in the percentage of altered mucosa in the rectum of the rats killed at 31 to 52 weeks compared with those killed at 20 weeks (Table 3).

None of the control rats (n = 30) killed 20 to 52 weeks after

Table 1 Proportion of foci identified with alterations of each of the several phenotypes in rats killed at different time periods after the first injection with DMH

<table>
<thead>
<tr>
<th>Altered phenotype*</th>
<th>20 weeks</th>
<th>31–52 weeks</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hex, d epithelial</td>
<td>76</td>
<td>71</td>
<td>74</td>
</tr>
<tr>
<td>ANBE, d epithelial</td>
<td>68</td>
<td>71</td>
<td>69</td>
</tr>
<tr>
<td>PAS, d epithelial</td>
<td>56</td>
<td>82</td>
<td>67</td>
</tr>
<tr>
<td>GGT, i stromal</td>
<td>64</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>ALP, i stromal</td>
<td>48</td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td>5’N, d fibroblast sheath + i stromal</td>
<td>36</td>
<td>53</td>
<td>43</td>
</tr>
<tr>
<td>ACP, d epithelial + i stromal</td>
<td>32</td>
<td>65</td>
<td>45</td>
</tr>
<tr>
<td>Hex, d, ANBE, d, PAS, d</td>
<td>28</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td>Hex, d, ANBE, n, PAS, d</td>
<td>20</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Hex, d, ANBE, d, PAS, n</td>
<td>20</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Hex, n, ANBE, d, PAS, n</td>
<td>16</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Hex, n, ANBE, d, PAS, d</td>
<td>4</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
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<td>4</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Hex, n, ANBE, n, PAS, n</td>
<td>8</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

* Histochemically demonstrable activities were decreased (d), not altered (n), or increased (i) as compared with normal adjacent tissue. Fibroblast sheath cells surrounding normal crypts have strong 5'-nucleotidase (5’N) activity but are absent or lack 5'-nucleotidase activity in enzyme-altered foci. Hex, hexosaminidase; ALP, alkaline phosphatase; ACP, acid phosphatase.
their first injection or experimental rats \( (n = 12) \) killed 20 weeks after their first injection with DMH. On the other hand, 5 of 20 rats killed 31 to 52 weeks after their first injection with DMH had grossly identifiable lesions. The only lesion located in the proximal colon was identified as a lymphoid aggregate; the remaining six tumors in five rats were identified as colonic carcinomas in histological sections stained with HEA. One tumor was located in the mid colon, four in the distal colon and one in the rectum. The weights and weight gains of the control and experimental rats that received the relatively low dose of DMH that we used were not significantly different (Table 4).

**DISCUSSION**

The aim of this study was to identify phenotypic marker(s) of putative preneoplastic colonic cells that would identify cells very early in the carcinogenic process, before the expression of morphological changes that could be identified grossly or by routine histological stains. Consistent with this goal, high doses of carcinogen that are known to cause extensive alterations were avoided. The enzyme histochemical changes that are described in this paper fit that criterion, i.e., the focal histochemically demonstrable changes were observed in areas that were morphologically normal as well as in some that were abnormal. The increased numbers and areas of enzyme-altered foci in the colons of rats treated with carcinogen compared with control rats are consistent with the hypothesis that these foci are related to the carcinogenic process; however, these data do not rule out the possibility that colonic enzyme-altered foci are epiphenomena in carcinogen-treated rats. Additional experiments are needed to determine their relationship to the carcinogenic process. The persistence of these foci in the colons of rats several months after the termination of treatment with DMH further suggests that focal enzymatic alterations are often permanent and not acute manifestations of the toxic effects of the carcinogen. It should be noted that, although enzyme-altered foci were identified in all of the rats from which tissues were analyzed, we were unable to classify any samples as showing focal enzymatic alterations that would identify cells that were not overtly dysplastic; the focal enzymatic alterations described in this paper fit that criterion, i.e., the focal histochemically demonstrable changes were observed in areas that were morphologically normal as well as in some that were abnormal. The increased numbers and areas of enzyme-altered foci in the colons of rats treated with carcinogen compared with control rats are consistent with the hypothesis that these foci are related to the carcinogenic process; however, these data do not rule out the possibility that colonic enzyme-altered foci are epiphenomena in carcinogen-treated rats. Additional experiments are needed to determine their relationship to the carcinogenic process. The persistence of these foci in the colons of rats several months after the termination of treatment with DMH further suggests that focal enzymatic alterations are often permanent and not acute manifestations of the toxic effects of the carcinogen. It should be noted that, although enzyme-altered foci were identified in all of the rats from which tissues were analyzed microscopically at 31 to 52 weeks, this comprised only a small number of rats, i.e., six, that had received DMH for an extended period of time. Larger numbers of rats treated with carcinogen for shorter periods of time are needed to examine the possibility that these foci might be due to the toxicity of the chronic administration of carcinogen. Decreased hexosaminidase activity in morphologically normal and dysplastic lesions in our studies and in colonic tumors of DMH-treated rats in the studies of Freeman et al. (35) suggests that hexosaminidase will be a good marker to follow...
cells through the progression from normal to malignant. Increased GGT activity in the stroma surrounding many enzyme-altered crypts and biochemically increased GGT activity in DMH-induced colon tumors (39) suggest that GGT will also be a good marker to follow carcinogenesis. Histochemically demonstrable hexosaminidase and ANBE in the crypts of normal proximal colon have lower activity than that observed in the distal colon (16). The absence of high levels of these activities in proximal colon makes the use of decreases in these activities impractical for the identification of foci in the proximal colon. Additional markers more appropriate for the proximal colon are being sought. The lack of detection of altered foci in proximal colon in our preliminary experiments does fit the pattern of tumor development in DMH-treated rats and the observations of structurally altered crypts by McLellan and Bird (45) with a very different system.

DMH has been widely used in experimental animals for the induction of colon carcinomas (42, 46–57) that have many similarities to the human disease (48, 52, 53). The dose of 15 mg/kg DMH (6.8 mg/kg of the base, dimethylhydrazine) had the advantage of inducing only mild toxicity, as measured by weight gain, while still resulting in some tumor formation after an extended period of time (31 to 52 weeks). Having demonstrated that enzyme-altered foci develop early in the colons of rats that received a carcinogenic dose of DMH, it will be interesting to determine if enzyme-altered foci can be induced by a nontumorigenic dose of a carcinogen followed by a promoter, as in the liver system (reviewed by Peraino et al.; Ref. 11). Such a system could then be used to test whether high concentrations of compounds such as fentanyl diglycinerides (58) affect the promotion of putative preneoplastic enzyme-altered foci to malignancy. This system should also provide a useful model system to evaluate when and how various dietary compounds or drugs affect colon carcinogenesis. Compounds such as inositol hexaphosphate (59) and difluoromethylornithine (60) appear to affect the promotion of colon cancer, while ethanol (61) appears to act by a different mechanism. We postulate that the former compounds may cause a reversal of existing enzyme-altered foci, whereas ethanol would have no effect on foci.

Several types of alterations, including changes in mucin secretion and morphology, have been reported to occur in the colons of animals treated with carcinogen, prior to the development of tumors (17, 32–34). None of these studies described the mucin changes as being focally discrete areas surrounded by normal activity, as we have described. The antigen M1 has been detected in individual cells of altered crypts during colon carcinogenesis in the rat (42). Focal areas of altered activity of several enzymes involved in carbohydrate metabolism as well as acid phosphatase were reported by Mayer et al. (12), but all of their illustrated lesions exhibited marked morphological abnormalities as well. Proliferative changes of colonic crypt cells after DMH treatment have also been linked with the carcinogenic process (62, 63).

With 1% alcian blue solution, Kimura et al. (64) detected abnormalities on the surface of rat colonic mucosa, after DMH treatment, that were classified as "mild, moderate, or severe dysplasia." Their lesions with mild dysplasia, as illustrated, are clearly more altered morphologically than the lesions we detected. More recently a similar protocol with 0.2% alcian blue has been used to detect altered crypts at various doses and times after carcinogen treatment in several strains of mice and rats (45, 65–67). Their altered foci contained between 1 and 2.17 altered crypts/focus, and in all studies "an aberrant crypt is at least 3 to 4 times larger than a normal crypt" (45).

Sandforth et al. (68) used a dissecting microscope to identify lesions on the surfaces of colonic segments from DMH-treated rats after mucosal staining with Schiff reagent. The altered areas contained between 1 and 7 crypts, altered cell proliferation, some enzymatic changes, and "mild epithelial dysplasia." These systems with unsectioned colon have the advantage that they allow the entire colon to be surveyed rapidly, but it seems unlikely that many of the enzyme-altered areas that we observed would be detected with these systems, since many of our foci do not have any apparent morphological abnormalities and most are not larger than normal crypts. The longitudinal sections of altered mucosa illustrated by Sandforth et al. (68) look like "microadenomas," i.e., the glands are adenomatous and elevated above the surrounding crypts. It seems likely that these lesions are precursors of adenomas and ultimately carcinomas, as discussed by Sandforth et al. (68) and McLellan and Bird (45). However, there are data that support the view (51) that there is "strong evidence that most experimental adenocarcinomas . . . arise de novo in flat mucosa, i.e., without a prior adenoma stage [in DMH-treated rats] . . . [and] most DMH-induced tumors in mice were . . . adenomas." It is likely that each system (aberrant crypts and enzyme-altered foci) detects (a) lesions that contain putative preneoplastic cells, (b) many of the same lesions, and (c) a population of preneoplastic cells that could not be detected by the other system. Together, these systems should provide additional means to elucidate more precisely the pathogenesis of colonic carcinoma.

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