Sequential Analyses of the Growth and Morphological Characteristics of Aberrant Crypt Foci: Putative Preneoplastic Lesions

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ABSTRACT

The main objective of the present study was to sequentially analyze growth and morphological characteristics of aberrant crypt foci (ACF) in the rat colon. Sprague-Dawley rats were given a single injection of a carcinogenic dose of 1,2-dimethylhydrazine-HCl and at varying time points ranging from 2 to 57 weeks, groups of 5 rats were terminated. The number and crypt multiplicity of ACF were determined in the distal 8 cm of the colon. In addition, ACF were processed for histology and then graded for the presence of nuclear atypia using a score of 0-4. The findings of the present study demonstrated that ACF exhibit the characteristics expected for precursor lesions. ACF were present at all time intervals in large numbers in the colons of rats treated with 1,2-dimethylhydrazine-HCl and were present when adenocarcinomas were observed. The number of ACF with 4 or more crypts and those exhibiting a higher grade (grade 4) of nuclear atypia increased significantly at or beyond 19 weeks. These features of ACF, particularly the presence of nuclear atypia indicative of dysplasia, provide strong support for the hypothesis that ACF are precursor lesions of chemically induced colon cancer.

INTRODUCTION

Bird (1) described a method that allows the examination of crypts on the mucosal surface of fixed, methylene blue-stained, unsectioned rodent colons under low magnification. Using this method, colons of mice and rats treated with a colon carcinogen are observed to contain crypts that are altered with respect to size and shape of the lumen and thickness of their epithelial lining compared with the surrounding normal crypts. These crypts, termed aberrant crypts, are present in groups of one or more forming distinct foci. We have hypothesized that ACF1 are precursor lesions of chemically induced colon cancer. Data on the induction and growth characteristics of ACF are consistent with this hypothesis (1-5). Furthermore, it has also been demonstrated recently, by using the methodology described by us (1), that aberrant crypts are enzyme-altered and are also present in human colons (6, 7). The present method to visualize ACF does not yield any specific information on the histological features of these lesions. Whether ACF are precursor lesions of colon cancer can be better established by sequentially studying their histological features.

Our method of viewing the whole colonic mucosa and identifying and quantifying ACF is simple. Therefore, if ACF are indeed precursor lesions of colon cancer, then this method could be readily used to study the stepwise development of colon cancer at the cellular and molecular level, and the number and growth of ACF could be used as a disease endpoint in the study of modifiers of colon carcinogenesis (1-5).

Strong evidence in support of the hypothesis that ACF are precursor lesions of colon cancer can be achieved by histologically examining the ACF for the presence of dysplasia or cancerous changes. It has been demonstrated that colonic mucosa of animals repeatedly treated with a colon carcinogen exhibit cytokinetic and histological changes at the crypt level including altered proliferative indices, crypt height and width, with or without dysplasia (8-13). Whether all these changes are specific to the carcinogenic process is not known. However, presence of dysplasia in a crypt is considered to be a precancerous feature in animal and human colons.

The main objective of our study was to investigate sequentially, number, crypt multiplicity, and histological features of ACF in rat colon after a single carcinogenic dose of DMH. Based on our knowledge of the pathogenesis of colon cancer and the nature of precursor lesions in other organs, one would expect precursor lesions of chemically induced colon cancer to exhibit at least the following 4 characteristics if one were to study the development of the disease with time. (a) More lesions than cancers should develop, since cancer is a multi-step process in which only a small number of lesions at each stage evolve to the next step (13, 14). (b) Some lesions should persist and be present when cancer develops. This has been observed in the skin and liver (14, 15). (c) The number of crypts per precursor lesion should increase with time based on the concept that colon cancer evolves from a single crypt (8-12). (d) Some lesions should exhibit dysplasia, inasmuch as it is considered to be important in the cancer process. In the present study, ACF was assessed with respect to these 4 characteristics.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Charles River Canada Inc., Montreal, Quebec, Canada), approximately 6-7 weeks old, were used. They were housed in plastic cages with wire tops and sawdust bedding with a 12-h light-dark cycle. The animals were fed Purina Laboratory Chow ad libitum and had free access to chlorinated water (5-10 ppm).

Study Design. The animals were given a single i.p. injection of 125 mg DMH (Sigma Chemical Co., St. Louis, MO) per kg body weight. The DMH was dissolved in 1 mM EDTA and the pH of the solution was brought to 6.5 by the addition of NaOH. Doses of DMH ranging from 40 to 150 mg/kg body weight given in a single injection have been reported to induce colon cancer in laboratory rats (16, 17). Based on these studies, we used a single injection of 125 mg DMH-HCl per kg body weight to induce colon cancer. Initially it was decided to kill 5 animals at 2 weeks, then at 6-10-week time intervals. It was expected that some animals would develop macroscopic lesions by 20 weeks and would develop cancer by 40 weeks. However, this did not occur. To ensure the presence of cancer, animals’ feces were examined for the presence of blood routinely. The last termination point, 57 weeks, was chosen when the presence of blood was quite evident in the fecal samples. Therefore, 5 animals were killed by carbon dioxide asphyxiation 2, 6, 12, 19, 32, 41, and 57 weeks after receiving the carcinogen...
injection. Their colons were removed, flushed with Kreb's-Ringer solution, slit open from cecum to anus, and fixed flat between 2 pieces of filter paper in 10% buffered formalin. Glass microscope slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. After a minimum of 24 h in formalin, the colons were placed in a solution of 0.2% méthylène blue (Sigma) dissolved in Kreb's-Ringer solution for 5 to 15 min, placed mucosal-side-up on the slide, and viewed with a light microscope.

Features of a crypt that can be identified by viewing the colons in this manner include the size and shape of the crypt, the thickness of the epithelium lining, and the size and shape of the luminal opening (1–5).

Criteria used to define an ACF consisting of a single crypt are as follows. The size of the crypt is at least twice that of the normal surrounding crypts; the luminal opening is more elliptical than circular; and the thickness of the epithelial lining is greater than that of the normal surrounding crypts.

ACF consisting of more than one crypt are characterized as follows: (a) individual crypts comprising the ACF have a thicker epithelial lining and an elliptical luminal opening; (b) the crypts have the appearance of forming a distinct focus, i.e., there are no normal-appearing crypts separating the crypts within the ACF; and (c) the total area occupied by the crypts composing the ACF is greater than the area occupied by an equivalent number of surrounding morphologically normal crypts.

Quantification of ACF. At all time points, except at 57 weeks, the number of ACF and number of crypts in each ACF in the distal 8 cm of the colons, starting from the rectal end, were recorded. ACF were not quantitated at 57 weeks because 2 of the 5 rats had large tumors that prevented accurate assessment of the number of ACF.

Histological Analysis of ACF. At all time points, except at 57 weeks, 2 to 6 ACF from between the 5th and 6th cm of each of the fixed colons measured from the rectal end were included for histological analysis. The ACF were from between the 5th and 6th cm of the colon measured from the rectal end. Under light microscopy, a microfeather scalpel was used to excise the ACF of interest and the surrounding normal crypts. The dissected tissue was 2 mm x 2 mm. The tissue was embedded in paraffin wax and serially sectioned at 5-μm sections cut parallel to the muscularis mucosa and stained with hematoxylin and eosin. The histology of each ACF was assessed for crypt architecture and nuclear features by comparison with the normal surrounding crypts.

Nuclear Features. The nuclear features of the ACF were assessed to determine whether or not the lesions exhibited dysplasia, a morphological alteration considered to be specific to the cancer process (18–20). In doing so, it was observed that at early time points, the majority of ACF did not exhibit features that could be classified as dysplasia using the definition of Konishi and Morson (20), and yet there were changes in the nuclear morphology of most ACF. It was apparent that a yes/no grading system for the presence of dysplasia would not provide maximum information concerning nuclear features of ACF. Further analysis of the ACF revealed that the nuclear features, which varied from ACF to ACF, were proportional to the ratio of elongated and stratified nuclei. Thus, a grading system based on these 2 features was devised (explained in Table 1) to describe nuclear atypia. The highest grade (grade 4) ACF could be classified as exhibiting nuclear features indicative of dysplasia. Using this grading system (Table 1), a nuclear grade was assigned to each ACF. Because the grade of nuclear morphology sometimes varied between crypts in an ACF and even within a crypt, the grade assigned to each ACF was the highest grade exhibited among all sections through the ACF. A similar approach is used in the grading of adenomas, where the degree of dysplasia has been observed to vary within these lesions (18–20). Histological features of ACF exhibiting nuclear grades of 0–4 is presented in Fig. 1. It was evident that the nuclear atypia noted in some ACF were similar to those seen in the crypts of adenocarcinomas present in the rat colons (Fig. 2).

Statistical Analysis. Statistical analysis of the data was performed by analysis of variance and Duncan's multiple range test. A probability of less than 5% (P < 0.05) was considered significant.

### Table 1 Numerical grading system to assess the nuclear morphology of ACF

<table>
<thead>
<tr>
<th>Nuclear stratificationa</th>
<th>None (0)</th>
<th>Mild (1)</th>
<th>Moderate (2)</th>
<th>Severe (3)</th>
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<tr>
<td>Nuclear elongationa</td>
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<tr>
<td>Mild (1)</td>
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<td>Moderate (2)</td>
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<td>Severe (3)</td>
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a Nuclear elongation, nuclei that had an elongated shape polarizing towards the crypt lumen; None, elongated nuclei not present or not to an extent greater than that observed in normal surrounding crypts; Mild, 1 to 2 regions in a crypt contain 2–3 elongated nuclei; Moderate, majority of nuclei elongated within the crypt; Severe, all nuclei elongated; 0, 1, 2, 3, or 4, numerical value assigned to each of the 4 levels of nuclear elongation.

b Nuclear stratification, elongated nuclei that do not line up against the basement membrane; None, no stratified nuclei present; Mild, 1 to 2 areas in a crypt contain 2–3 elongated nuclei, of which 1–2 are stratified; Moderate, stratified nuclei present throughout most of crypt; Severe, stratification of nuclei throughout crypt; 0, 1, 2, 3, or 4, numerical value assigned to each of the 4 levels of nuclear stratification.

c —, ACF with these combinations of features were not observed.

d — ACF with these combinations never occurred because stratified nuclei are also elongated nuclei.

### RESULTS

Macroscopic lesions were not observed in the colons of any animals terminated before the 57-week point. Two of the 5 rats terminated at 57 weeks had invasive adenocarcinoma measuring 1 to 2 cm in diameter.

The effect of time on the number of ACF in the distal 8 cm of the colon is presented in Fig. 3. After a significant rise in the number of ACF between 2 and 6 weeks after carcinogen treatment, the number decreased significantly by 19 weeks. This was followed by a striking increase at 32 and 41 weeks. Although not quantitated at 57 weeks, ACF were present in all 5 colons including the 2 colons that contained adenocarcinomas. All colons analyzed at each time point from 2 weeks to 57 weeks contained ACF. The smallest number of ACF quantitated in the distal 8 cm of a colon was 13, in a rat killed at 2 weeks after carcinogen administration.

The colons of rats examined at 2 weeks post-treatment contained ACF consisting mainly of 1 or 2 crypts. At 6, 12, 19, 32, and 41 weeks, the greatest number of crypts comprising an ACF was 8, 8, 14, 30, and 43, respectively (data not presented). The percentage of ACF consisting of 4 or more crypts was significantly greater at 19, 32, and 41 weeks after carcinogen treatment than at any of the earlier time points (Fig. 4).

The crypts comprising the ACF are larger than the normal surrounding crypts. This parameter varied in relation to the depth of the section taken. In sections close to the luminal surface, ACF crypt area was generally 2 to 3 times larger than normal surrounding crypts, whereas near the muscle layer the difference was only 1.5 times or less. Furthermore, in sections close to the luminal surface, ACF crypts usually exhibited an elliptical shape. However, as the sections progressed towards the muscle layer, some ACF crypts maintained their elliptical shape, whereas others became more circular or tortuous. The significance of the variable crypt shape of ACF is not known. However, it should be noted that the crypt architecture of ACF as they appear in histological sections near the luminal surface is consistent with the appearance of ACF crypts when viewing the mucosal surface of the colon with the methylene blue method.

The percentage of ACF exhibiting each of the 5 grades of nuclear morphology is presented in Fig. 5. From the results, it is evident that percentage of ACF exhibiting nuclear atypia of
GROWTH AND MORPHOLOGY OF ABERRANT CRYPT FOCI

Fig. 1. Photomicrographs of ACF exhibiting grades of nuclear morphology from colons of rats receiving a single injection of 125 mg DMH per kg body weight. All sections were cut parallel to the muscle layer. A, grade 0. ACF consisting of 9 crypts from 41 weeks after carcinogen treatment. Note the generally circular shape of the nuclei and the absence of stratification. H & E, x 250. B, grade 1. ACF consisting of 28 crypts from colon 41 weeks after carcinogen treatment. Note the presence of a few elongated nuclei and the absence of stratified nuclei. H & E, x 250. C, grade 2. ACF consisting of 13 crypts 19 weeks after carcinogen treatment. Note the presence of elongated nuclei scattered throughout the crypts and the absence of stratified nuclei. H & E, x 250. D, grade 3. ACF consisting of one crypt 2 weeks after carcinogen treatment. Note the presence of elongated nuclei scattered throughout the crypt and the absence of few stratified nuclei. H & E, x 250. E, grade 4. ACF consisting of 6 crypts 32 weeks after carcinogen treatment. Note the similarity of the nuclei in these crypts and those from the 2 adenocarcinomas is displayed in Fig. 2, C and D. H & E, x 250. F, 2 ACF 41 weeks after carcinogen treatment. ACF with 10 crypts exhibits a grade of 0, whereas the ACF with 21 crypts exhibited a grade of 3. H & E, x 25.

The highest grade increased markedly at or beyond the 19-week time point.

There was no apparent effect of time on the nuclear grade of ACF consisting of varying numbers of crypts (Table 2). Also, no apparent association was found between the number of crypts per ACF and nuclear grade of ACF at a given time after carcinogen treatment.

DISCUSSION

ACF were studied in the colons of rats terminated at various time points after a single injection of a carcinogenic dose of DMH and assessed for total number of crypts and morphological atypia.

The mean number of ACF per the distal 8 cm of rat colon ranged from 13 at 2 weeks to a high of 160 at 41 weeks after carcinogen treatment. ACF were present in all colons analyzed at each time point, including 57 weeks, at which time 2 of the 5 colons contained adenocarcinomas. The finding that ACF are present in colons at all time points before and including the appearance of cancer, suggests that at least some ACF persist and may be the site of cancer development. Presently, it is not clear why the number of ACF decreased at 12 and 19 weeks and then markedly increased at 32 and 41 weeks. However, this decrease and subsequent increase seen in the total number of ACF were observed mainly due to a decrease or increase in the number of ACF consisting of 1, 2 or 3 crypts (data not presented). This finding suggests that some ACF are transient in nature and that they either are eliminated or undergo phenotypic reversion, a well-documented observation with respect to the putative preneoplastic lesions in the liver model (14, 15, 21). It is noteworthy that focal areas of atypia implicated as putative preneoplastic lesions also regress or undergo phenotypic reversion (22). Our observation that a reduction and then an increase in the number of ACF generally consisting of ACF with 1 or 2 crypts is interesting and raises important questions regarding the developmental aspects of ACF and their importance in colon carcinogenesis. Confirmation of the observation that some ACF are indeed transient lesions is needed before any significance is attached to this particular finding.

Division of data into ACF consisting of 1, 2, 3, or 4 or more crypts revealed that the number of ACF consisting of 4 or more crypts was significantly greater at the later time points. This suggests that the number of crypts per ACF increases with time in a large proportion of ACF and is consistent with the hypothesis that ACF undergo crypt multiplication and/or crypt branching.

A majority of ACF exhibited morphological atypia starting at 2 weeks. Using a grading system to enumerate atypia based on nuclear morphology (elongation and stratification), it became evident that some ACF exhibited a grade 4 nuclear atypia around 19 weeks. Nuclear elongation and stratifications are the 2 main characteristics of dysplastic epithelium (18–20).

Because dysplasia is generally considered to be a significant finding in the carcinogenic process (18–20), our observation that some ACF exhibit dysplasia presents strong evidence that ACF represent precursor lesions. An ACF exhibiting mild dysplasia (Fig. 1F) revealed nuclei with the characteristic "picket-fence" pattern seen in human adenomatous polyps in association with mild dysplasia (18, 19). However, the observation that not all ACF exhibited dysplasia raises interesting questions. If dysplasia can be used as a basis for distinguishing...
ACF with an increased neoplastic potential from those with no increased potential, the histological appearance of ACF expressed at the time the lesions are formed (i.e., at the single crypt stage) would be of paramount importance. If this is not the case (i.e., if dysplasia can be expressed at later time points), then dysplasia could not be used as a marker of neoplastic potential. In the present study, due to the small sample size per time point, we were unable to demonstrate statistically whether or not dysplasia increases with time. However, we did observe that the histopathology of ACF varies and that there is a progression from lower to higher grades of nuclear changes with time that allowed us to devise a grading system.

To establish a quantitative relationship between number of crypts per ACF and grade of dysplasia, and between nuclear grade (dysplasia) and time, histological features of a large number of ACF with varying crypts numbers at various times will need to be examined. It is essential to establish whether these relationships exist.

From our findings, it is apparent that quantification of ACF
in the whole colon includes a majority of lesions with varying grades of nuclear atypia indicative of dysplasia. Presence of dysplasia is regarded as early histopathological changes in the precursor lesions of colon cancer (22), however, at the genetic level it may be considered a late event (23). Therefore, the time and appearance of dysplasia in ACF will provide us with a better understanding of the genesis of colon cancer and role of ACF in this process.

In light of the present findings that a carcinogenic dose of DMH induces a large number of ACF, ACF grow with time, several ACF are present in the epithelium when cancers appear, and most importantly some ACF exhibit dysplasia, an important feature of precursor lesions of colon cancer, we conclude that ACF are indeed precursor lesions of chemically induced colon cancers.

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**REFERENCES**

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