ABSTRACT

Murine colons treated with the colon carcinogen azoxymethane (AOM) have been reported to contain aberrant crypts (AC), which are characterized by their larger size and wider pericryptal zones. The methodology used to visualize AC consists of staining the fixed, unsectioned colonic mucosa with methylene blue and transillumination of the luminal surface at a magnification of 40x. The objective of the present studies was to determine if AC demonstrate characteristics to support our hypothesis that they are putative preneoplastic lesions. Studies were designed to determine the time of occurrence of AC (Study I), the induction of AC in response to varying dosages of AOM ranging from 0.0 to 10.0 mg/kg body weight (Study II), and the effect of a high fat diet (20% fat by weight) on the number and size of AC (Study III). In all studies 4-6-week-old female CF, mice were used. In addition C57BL/6J female mice were used in Study I. The major findings were as follows: (a) the time period required to form AC was approximately 2 weeks following a single AOM injection (5 mg/kg); (b) a dose-dependent increase in the induction of AC was noted in response to AOM from none in the control group to a plateau level of 2.90 ± 0.38 foci at a dose of 5.0 mg/kg; (c) in comparison to the low fat group, the high fat group had a greater (P < 0.05) mean number of foci of AC per 5 cm of colon (15.67 ± 1.32 vs. 11.44 ± 1.44) and a larger (P < 0.05) mean size of foci of AC (0.0296 ± 0.0012 mm vs. 0.0249 ± 0.0012 mm) after 16 weeks on the respective diets; and (d) preliminary histological appearance of foci of AC revealed mild atypia to unequivocal dysplasia. The findings of the present study are consistent with the hypothesis that AC are putative preneoplastic lesions.

INTRODUCTION

Our understanding of the histogenesis of colon cancer has come from sequential morphological studies done in both humans and animals. Based on such studies it is believed that colon cancer, like the majority of cancers, evolves from precursor lesions (1). Work done in humans by Morson and his colleagues (2, 3) and by Lane and colleagues (4, 5) provides strong support for the contention that colon cancer evolves from adenomatous polyps. To identify the possible precursor lesions of adenomatous polyps, and hence the early precursor lesions of colon cancer, several investigators have studied the development of colon cancer in rodents treated with chemical carcinogens.

Generally, the protocol employed in these studies consists of treating the animals with multiple injections of the carcinogen and then viewing serial, histological sections of the colon in order to note any changes. Following such a protocol several researchers (6, 7) have identified crypts with distinctly altered proliferative patterns. They have proposed that such crypts represent one of the early precursor lesions in the evolution of colon cancer. In addition, Chang (8), following a similar protocol, noted single crypts exhibiting dysplasia which he has proposed represent preneoplastic lesions.

The above-mentioned studies, by identifying putative preneoplastic lesions, have contributed greatly to our understanding of the stages involved in the development of colon cancer. However, the methodology used to identify these precursor lesions does not allow for quantification of the lesions. In fact, at the present time there is not a methodology capable of identifying and quantitating the early putative preneoplastic lesions in the colon. Without such a methodology it is not possible to employ these lesions as endpoints in studies.

Recently, in our laboratory we have developed a methodology which may be capable of identifying and quantitating the early preneoplastic lesions in the rodent colon (9). By applying this methodology we have observed lesions at the crypt level, present in the colons of rodents treated with a colon carcinogen. The cryptal lesions are distinguished by their increased size, thicker epithelial lining, and increased pericryptal zone. We have termed these lesions AC. Since AC have only been observed in the colons of carcinogen-treated rodents and never in the colons of control rodents, we have hypothesized that AC may represent early preneoplastic lesions in the rodent colon.

Research in our laboratory is aimed at determining whether or not AC demonstrate characteristics to suggest they may be preneoplastic lesions. The purpose of this paper is to report on our findings from three such studies. Study I was designed to determine the time of occurrence of AC and number of AC induced in response to one injection of the colon carcinogen, AOM, in two strains of mice differing in their sensitivity to developing colon cancer. The objective of Study II was to determine if there is a dose-dependent response between the number of AC formed and the dosage of AOM given. The effect of a HF diet on the growth of AC was investigated in Study III. Preliminary findings on the histological appearance of AC are also presented.

MATERIALS AND METHODS

Animals. In all studies the animals were housed in plastic cages with wire tops and sawdust bedding in a 12-h light-dark cycle. Unless otherwise stated, the animals were fed AIN pellets (10) ad libitum and had free access to water.

Visualization and Quantification of AC. Immediately following termination of the animal, the colon was removed, flushed with Kreb's Ringer, slit open from cecum to anus, and fixed in 10% buffered formalin. Following the protocol cited by Bird (9), the fixed colons were stained with methylene blue and then using the light microscope the colons were assessed for AC. The parameters used to assess the colons were occurrence, size, and distribution of AC. The occurrence was measured by quantitating the mean number of foci of AC per colon. The number of AC per focus was also recorded. To assess the size of the foci, a grid was placed in the eyepiece of the light microscope and the number of AC was counted. The size of AC was determined by measuring the diameter of the AC at the point of maximum width using a micrometer eyepiece. The size was expressed as the mean of the maximum and minimum diameters.

Preliminary findings on the histological appearance of AC are also presented.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: AC, aberrant crypts; AOM, azoxymethane; b.w., body weight; HF, high fat; LF, low fat.
divided into two groups of 20 mice each. Group 1 received a single i.p. injection of 5 mg AOM (Ash Stevens) per kg b.w. Group 2 acted as the control group and received a single i.p. injection of saline. Five mice from each group were terminated by cervical dislocation, 1, 2, 3, and 4 weeks after receiving the injection.

Study II: Dose Response. 4-week-old CF¡ female mice were randomly divided into six groups. Each group received one i.p. injection of AOM at one of the following dosage levels: 0.0, 1.0, 2.5, 5.0, 7.5, or 10.0 mg per kg b.w. 2 weeks after the carcinogen treatment the mice were terminated by cervical dislocation.

Study III: Dietary Modulation. 4-week-old CF¡ mice were randomly divided into two groups. Group 1 received 4 weekly i.p. injections of 5 mg AOM per kg b.w. Group 2 received 4 weekly i.p. injections of saline. 1 week following the fourth injection half the mice were fed a HF diet (15% beef tallow and 5% corn oil by weight) while the remaining continued on a LF diet (5% corn oil by weight). The compositions of the diets are listed in Table 1. To make the HF diet extra fat was added to the LF diet at the expense of carbohydrate. The nutrient per kilocalorie density of the HF diet was identical to the LF diet except with respect to fat and carbohydrate. The mice were terminated at either 0, 4, 8, or 16 weeks on the diets.

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.

RESULTS

No AC were identified in the colons of mice given saline alone. Therefore, only the data for the mice treated with carcinogen are presented in the tables below.

Study I: Time of Occurrence. The results of Study I are shown in Table 2. In both CF¡ and C57 mice, the AC were not detectable until 2 weeks following the AOM injection. At the 2-week termination point CF¡ mice had a significantly higher mean number of foci per colon compared to C57 mice at the same time point. The mean number of foci per colon in CF¡ mice tended to decrease with time and the opposite trend was noted in C57 mice. Due to the small number of mice per group, neither trend was statistically significant. It is interesting to note that the CF¡ mice showed 100% incidence of AC throughout the study. However, with the C57 mice, it was not until the 4th week that the 100% incidence level was reached. There were no significant changes in the size of foci or the number of AC per focus in either strain as a function of time.

Study II: Dose Response. There was no significant difference between the mean weights of the six groups (data not shown). The AC data for Study II is presented in Table 3. It is evident that there is a trend of increasing number of foci of AC per colon in response to increasing dosages of AOM up to 7.5 mg of AOM after which there appears to be a leveling off. A similar trend is also seen with the size of the foci as well as the number of AC per focus in response to the dosage of AOM given.

Although the majority of AC are located towards the rectal end, it is apparent that the higher dosages of AOM (7.5 and 10.0 mg/kg b.w.) tended to induce a relatively higher number of AC towards the cecum end than the lower dosages.

Study III: Dietary Modulation. The body weights of the animals on the HF diet tended to be higher than those on the LF diet. However, there was no significant difference between the mean weights of the mice on the HF and LF diets at any time point in the study (Table 4). The AC data for Study III are listed in Table 5. The HF group had more foci of AC per colon compared to the LF group at each of the respective time points. However, the difference was statistically significant only at the 16-week point. The mean size of the foci was significantly larger in the HF group compared to the LF group at the 16-week point. Between the 8th and the 16th week there was a significant increase in the mean number of foci per colon and in the mean number of AC per focus within both treatment groups. Also, the mean size of the foci increased in both treatment groups between the 8th and the 16th week although it was only statistically significant in the HF group.

Appearance and Distribution of AC. Foci of AC are easily recognizable by their increased size, pericryptal area, and thicker epithelial lining. Fig. 1A, B, D, and E, depict foci of AC present in unsectioned, methylene blue-stained, carcinogen-treated murine colons. Foci of AC containing varying numbers of AC have been observed. Fig. 1C shows the histological appearance of the focus identified in Fig. 1B. Examination of

Table 1 Percentage by weight composition of the diets

| Casein | 20.0 | 22.7 |
| Dextrin | 15.0 | 10.5 |
| Sucrose | 50.0 | 35.0 |
| Corn oil | 5.0 | 5.0 |
| Beef tallow | 15.0 | 10.5 |
| AIN-76 mineral mix | 3.5 | 4.1 |
| AIN-76 vitamin mix | 1.0 | 1.2 |
| α-Celullose | 5.0 | 5.9 |
| dl-methionine | 0.3 | 0.4 |
| Choline bitartrate | 0.2 | 0.2 |
| % of calories from linoleic acid | 7.1 | 7.0 |

* AIN-76 diet.
* Modified AIN-76 diet.

Table 2 Effect of time on appearance of AC in murine (CF¡ and C57BL/6J) colon induced by azoxymethane

<table>
<thead>
<tr>
<th>No. weeks</th>
<th>Incidence</th>
<th>No. foci/colon</th>
<th>No. AC/focus</th>
<th>Size of foci (× 10^-2 mm²)</th>
<th>% Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF¡ mice</td>
<td>0/5</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5/5</td>
<td>4.00 ± 1.30</td>
<td>1.00 ± 0.00</td>
<td>1.65 ± 0.11</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>5/5</td>
<td>2.60 ± 0.08</td>
<td>1.23 ± 0.21</td>
<td>1.88 ± 0.21</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>5/5</td>
<td>3.00 ± 0.20</td>
<td>1.36 ± 0.13</td>
<td>1.98 ± 0.11</td>
<td>71</td>
</tr>
<tr>
<td>C57BL/6J mice</td>
<td>0/5</td>
<td>0.00 ± 0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2/4</td>
<td>0.50 ± 0.29</td>
<td>1.00 ± 0.00</td>
<td>1.25 ± 0.25</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>3/4</td>
<td>1.00 ± 0.41</td>
<td>1.00 ± 0.00</td>
<td>1.75 ± 0.14</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>5/5</td>
<td>2.60 ± 0.93</td>
<td>1.31 ± 0.13</td>
<td>1.50 ± 0.13</td>
<td>77</td>
</tr>
</tbody>
</table>

* No. of weeks between injection and termination.
* No. of colons with AC over total no. of colons scored.
* All values shown are the mean ± SE.
* Distribution of foci in the divisions of the colon. See text for explanation of abbreviations.
* Means with the same letter are not significantly different (P < 0.05).
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Table 3 Effect of various doses of AOM on number and size of AC in CF, mice

| Dosage (mg/kg b.w.) | Incidence | No. foci/colon | No. AC/focus | Size of foci (mm²) | % distribution
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0/10</td>
<td>0.00 ± 0/0</td>
<td></td>
<td>1.0 ± 0/0</td>
<td>100 0 0</td>
</tr>
<tr>
<td>2.5</td>
<td>1/9</td>
<td>0.11 ± 0.11</td>
<td></td>
<td>1.03 ± 0.03</td>
<td>83 17 0</td>
</tr>
<tr>
<td>5.0</td>
<td>10/10</td>
<td>2.90 ± 0.34</td>
<td></td>
<td>1.31 ± 0.06</td>
<td>67 29 4</td>
</tr>
<tr>
<td>7.5</td>
<td>18/19</td>
<td>4.05 ± 0.42</td>
<td></td>
<td>1.98 ± 0.09</td>
<td>56 32 12</td>
</tr>
<tr>
<td>10.0</td>
<td>17/18</td>
<td>3.17 ± 0.50</td>
<td></td>
<td>1.89 ± 0.13</td>
<td>56 32 12</td>
</tr>
</tbody>
</table>

* Dosages of AOM administered in mg per kg body weight.
* No. of colons with AC over total no. of colons scored.
* All values shown are the mean ± SE.
* % distribution of foci in the divisions of the colon. See text for explanation of R, M, and C abbreviations.
* Means with the same letter are not significantly different (P < 0.05).

Table 4 Mean body weights of CF, mice treated with azoxymethane and fed either a LF or HF diet for 4, 8, or 16 weeks

<table>
<thead>
<tr>
<th>Diet</th>
<th>4 weeks Body wt °, g of mice on experimental diets at 4 weeks</th>
<th>8 weeks Body wt °, g of mice on experimental diets at 8 weeks</th>
<th>16 weeks Body wt °, g of mice on experimental diets at 16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>25.36 ± 0.73</td>
<td>31.34 ± 1.34</td>
<td>34.00 ± 1.78</td>
</tr>
<tr>
<td>HF</td>
<td>25.86 ± 0.55</td>
<td>30.11 ± 1.05</td>
<td>36.52 ± 1.06</td>
</tr>
</tbody>
</table>

* Mean wt ± SE.

the histological appearance shows that the epithelial cells lining the AC appear to be disorganized. Fig. 1F depicts the histological appearance of the large and small foci of AC shown in Fig. 1E. It was apparent from the histological sections that the large focus of AC consisted of tortuous crypt structures manifesting dysplasia and hence could be termed a microadenoma.

Serial sections of the focus shown in Fig. 1C reveal that the larger crypt in the focus branches into two distinct crypts towards the muscularis mucosa (Fig. 2). Also, the pericryptal area of the AC shows accumulation of oval-shaped cells. The characteristics and significance of these cells are not known. It is noted that the AC contain mitotic figures whereas the surrounding normal crypts do not, thus suggesting that the zone of cell proliferation is increased in AC compared to normal crypts (Fig. 2E). Goblet cells are not as evident in AC compared to the surrounding normal crypts (Fig. 2, C–F).

The data from all four studies show that the majority of the AC are located in the distal colon. In addition, all studies show that an aberrant crypt is at least three to four times larger than a normal crypt. At a magnification of 100x, a normal crypt occupied 0.25–0.33 the area of one square on the measuring grid.

DISCUSSION

The results of Study I show that in CF, and C57BL/6J mice, AC require between 8 and 14 days to form following carcinogen treatment. This length of time seems reasonable given that histologically we have shown that AC have altered morphology. The finding that AC can be detected 2 weeks following carcinogen treatment is to our knowledge the earliest time a focal aberration at the crypt level has been demonstrated. It is also the first time such a lesion has been identified following a single, low dose treatment with a carcinogen. For example, following multiple carcinogen treatments, Deschner (11) has observed focal atypias confined to one or two crypts at 38 days.

The strain differences observed at the 2-week termination point in Study I would appear to indicate that C57 mice may be less sensitive to developing AC than CF, mice. However, by the 4-week termination point there was no significant difference between the two groups with respect to mean number of foci of AC per colon. Thurnherr et al. (12) have reported that CF, mice are more sensitive to developing colon tumors in response to exposure to the colon carcinogen, 1,2-dimethylhydrazine, than are C57 mice. Assuming CF, mice are indeed more sensitive and that AC are reflective of the colon carcinogenesis process, then the data reported in Study I would suggest that it is the rate at which AC are formed that is indicative of sensitivity to the development of colon cancer. Further studies are needed to assess this possibility.

The findings of Study II indicate that AC are induced in a dose-dependent manner following carcinogen treatment. The failure to show a pronounced dose response is likely due to the small differences between the dosages of carcinogen tested. Following a similar protocol, we have completed a dose-response study in Sprague-Dawley rats using dosages of the colon carcinogen 1,2-dimethylhydrazine ranging from 0.00 to 150 mg/kg b.w. The data generated from this study yielded a similar, yet more pronounced, dose-response curve with the maximum number of AC being formed in response to a dose of 125 mg/
Fig. 1. Topographic views of AC in unsectioned methylene blue-stained murine colon and their histology. In A, topographic view of mucosa of colon from animal treated with a single i.p. injection of AOM and then left for 2 weeks. A single AC is surrounded by normal crypts. Note the increase in size and density of the epithelial lining of the AC, x 40. In B, topographic view of mucosa of colon from animal treated with 4 weekly i.p. injections of AOM. A foci of 2 AC is surrounded by normal crypts, x 40. In C, histological appearance of the focus shown in Fig. 1B. Note the architecture of the AC exhibit a focal appearance and mild cellular atypia. H & E, x 100. In D, topographic view of mucosa of colon from animal treated with 4 weekly i.p. injections of AOM and then fed a high-fat diet for 16 weeks. Two foci of AC side by side. The smaller focus (arrow) consists of 2 AC. The number of AC in the larger focus could not be determined, x 10. In E, histological appearance of the two foci in Fig. 1E. Note that the two AC constituting the smaller focus are larger than the normal crypts (arrow). Dysplasia is present in the larger focus, H & E, x 20.

The findings that AC are induced in a dose-dependent manner suggests that there is a cause-and-effect relationship between formation of AC and exposure to the carcinogen and, hence, supports the hypothesis that AC represent preneoplastic lesions. It is interesting to note that the size of AC foci, as well as the number of AC per focus, showed a similar response to increasing doses of carcinogen as did the number of AC per colon. Although the trend noted within each of these two parameters was not statistically significant at 2 weeks after carcinogen treatment, it is possible that with time a statistically significant difference between response and dose of carcinogen would be observed for both these parameters. Study I showed that AC require between 8 and 14 days to form. Therefore, by just 2 weeks after carcinogen treatment the parameter most likely to demonstrate a dose response is the number of AC induced. However, once induced it is likely that with time the growth of the AC, as assessed by their number and size, is enhanced by a HF diet consisting of 20% fat by weight, 75% of which is beef tallow and 25% of which is corn oil. Several long term tumor incidence studies have shown that a diet in which 20% or more of the diet by weight is from corn oil, safflower oil, beef fat, lard, or any combination thereof, has a promoting effect on colon carcinogenesis (13–16). Since a HF diet has been shown to promote chemically induced colon carcinogenesis in the rodent model, it was reasoned that a HF diet should also promote the growth of AC, if AC are indeed preneoplastic lesions. Therefore, the finding of Study III showing that the growth of AC can be enhanced by a HF diet is further support for our contention that AC represent preneoplastic lesions. It is known that AC are not formed in response to a HF diet. Therefore, the increase in the mean number of foci of AC per colon with time that was observed in Study III must be due to the appearance of slower growing AC.

In the murine system the majority of tumors arise in the distal colon (17). Therefore, the observation that the majority of AC also occur in the distal colon is consistent with the possibility that AC are involved in colon carcinogenesis. The results of all studies show that the area occupied by the luminal opening of AC is three to four times that occupied by normal crypts. It was noted that one AC (Fig. 2) may have had...
a large luminal opening because it was multiplying. The histological appearance of two foci, both consisting of two AC (Fig. 1, C and F), is clearly atypical. Although the atypia seen in the smaller focus cannot be called unequivocally dysplasia, the larger focus in Fig. 1F clearly exhibits dysplasia and can be termed a microadenoma, which is considered to be a precursor lesion of colon cancer (18). When the unsectioned tissue was stained with methylene blue, the microadenoma looked as though it was composed of many AC. It is our contention that the microadenoma evolved from a single AC which at one time exhibited only mild atypia (such as the one in Fig. 2). If our contention is correct, we would have a simple method for the identification of very early preneoplastic lesions (i.e., "preneoplastic" crypts) right through to microadenomas. Such a methodology would greatly facilitate the study of the multistep process of carcinogenesis in the colon. To substantiate our hypothesis a systematic characterization of AC with time is being carried out in our laboratory.

The main objective of the studies reported in this article was to determine if the lesions we have identified, by the methodology developed in our laboratory, demonstrate characteristics to suggest that they may represent preneoplastic lesions in the rodent colon. Based on the results, the following is known about AC in the murine colon: they can be induced in 2 weeks by a low dosage of a colon carcinogen, they are induced in a dose-dependent manner, their growth is affected by dietary modifications, they are largely located in the distal colon, and they exhibit morphological alterations. All these observations are consistent with our hypothesis that AC represent preneoplastic lesions.

Further studies in the rodent model are in progress to sequentially characterize AC for morphological, proliferative, and histochemical changes.

The existence of a methodology capable of identifying and quantitating preneoplastic lesions in the rodent colon will serve as an important tool in extending our knowledge of the cellular events taking place during colon carcinogenesis. The existence of such a system for the study of experimentally induced skin and liver cancer has already contributed greatly to our understanding of the cancer process (1). Furthermore, induction of AC can be used as a screening system to identify colon carcinogens in our diet; and the growth of AC can be used to identify and assess dietary factors capable of modulating the development of colon cancer.

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POTENTIAL PRENEOPLASTIC LESIONS


Aberrant Crypts: Potential Preneoplastic Lesions in the Murine Colon

Elizabeth A. McLellan and Ranjana P. Bird