Flat Dysplastic Aberrant Crypt Foci Are Related to Tumorigenesis in the Colon of Azoxymethane-Treated Rat

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Abstract

We evaluated the role of aberrant crypt foci (ACF) as biomarkers of colon cancer by studying the sequential development (6–28 weeks) from early lesion to tumor in the colon of azoxymethane-exposed F344 rats (15 mg/kg bw × 2). Surface examination of unsectioned methylene blue–stained colon preparations, transilluminated in the inverse light microscope, revealed two types of early lesions: classic elevated ACF and small flat lesions, which we denoted flat ACF and which were characterized by bright blue staining, compressed crypt openings, and crypts not elevated above the surrounding mucosa. At a later stage, the crypts surrounding large flat ACF became enlarged, a change that slightly raised the structure; principally, large flat ACF and nascent tumors displayed the same surface morphology. Furthermore, flat ACF with 18.6 ± 10.6 crypt/focus and tumors showed a uniform picture of severe dysplasia with frequent presence of Paneth cells, compressed crypts, cytoplasmic/nuclear overexpression of β-catenin, and nuclear overexpression of cyclin D1. In contrast, classic elevated ACF with 5.3 ± 2.5 crypts/focus did not display such changes: they showed mainly hyperplasia, mild or moderate dysplasia but never severe dysplasia. Along the time course, the number of flat ACF + tumors, including microscopic and macroscopic, was virtually constant, ~2.5 lesions/rat. The number of classic elevated ACF was initially ~180 lesions/rat and terminally ~80 lesions/rat. Flat ACF grew significantly faster than classic elevated ACF. In conclusion, our data indicate a continuous developmental growth from small flat dysplastic ACF to the stage of a tumor. In contrast, classic elevated ACF do not seem to be as closely related to tumorigenesis. (Cancer Res 2005; 65(1): 121–9)

Introduction

Aberrant crypt foci (ACF) were described in 1987 by Bird and Good (1) as putative preneoplastic lesions in the colon of carcinogen-treated rodents. ACF are easily scored by light microscopic examination of formalin-fixed whole-mount colon preparations stained with methylene blue. ACF are defined by their characteristic morphology: the crypts are enlarged, they have thickened layer of epithelial cells, they have increased pericryptal space, they have irregular lumens, and they are microscopically elevated. ACF are identified in histologic sections and by scanning electron microscopy (2), and they have been observed in patients with colorectal cancer and in patients with familial adenomatous polyposis (3). Because the total number and size (crypt multiplicity) of these small lesions can be scored routinely, ACF have been used as a short-term bioassay to evaluate the role of nutritional components and chemopreventive agents at an early stage of colon carcinogenesis (4).

Although ACF may share morphologic, genetic, and biochemical features with colonic tumors (5, 6), the development of these lesions is not clearly related to the early development of tumors. In azoxymethane/dimethylhydrazine–treated rodents and in patients with sporadic colorectal cancer, the number of tumors is minuscule compared with the large number of ACF (3, 7), demonstrating that only a very small fraction of the ACF in theory has the potential to progress to the stage of a tumor. Furthermore, in azoxymethane/dimethylhydrazine–treated rodents, a negative correlation between ACF formation and tumor formation (7, 8) and a discrepancy between spatial distribution of ACF and spatial distribution of tumors were reported (9). Therefore, there is a strong need to clarify the role of ACF in colon carcinogenesis and to validate their relevance as biomarkers of tumorigenesis.

In Min/+ mice, an Apc/familial adenomatous polyposis model, we did not observe spontaneous formation of classic ACF (10), although these animals spontaneously develop adenomas in the colon. However, we discovered small flat dysplastic lesions, which we denoted ACFMin. In contrast to classic elevated ACF, these lesions were not elevated above the surrounding mucosa, and their detection by surface examination in whole-mount colon preparations were totally dependent on both methylene blue staining and transillumination. ACFMin exhibited dysplastic crypts similar to those found in adenomas, and like the adenomas, they responded to chemoprevention by dietary fish oil (11). In Min/+ mice exposed to azoxymethane, we observed additional ACFMin as well as classic elevated ACF (12). However, only the ACFMin showed a continuous development from the monocryptal stage to adenoma with fast crypt multiplication and altered control of β-catenin. In contrast, the classic elevated ACF were hyperplastic, slow-growing, and showed normal β-catenin expression, and they were probably not as directly related to tumorigenesis. The fact that azoxymethane treatment also led to the formation of ACFMin in the wild-type littermates indicated that these lesions are general precursors of adenomas in the mouse colon.

It has become increasingly apparent that the Wnt signaling pathway, which is normally involved in repressing differentiation during embryogenesis and in the post-embryonic regulation of cell positioning in the intestinal crypts, is involved in tumor formation when aberrantly activated (13). Inactivating APC/Apc mutations or activating β-catenin mutations, which mimics Wnt stimulation and leads to β-catenin accumulation, is observed in the majority of colon cancers in both humans and rodents (14–17). Altered β-catenin expression is observed in small dysplastic lesions (18)

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and in dysplastic ACF (19). It is noteworthy that inactivation of both APC/Apc alleles is sufficient for the growth of early colorectal adenomas (20). Loss of APC function precludes the post-translational down-regulation of β-catenin, which consequently accumulates in cytoplasm and translocates into the nucleus where it complexes with the transcription factor Tcf-4 and activates specific target genes such as c-MYC and cyclin D1. Apparently, this gene activation represents a potentially oncogenic pathway.

The objective of the present work was to evaluate the role of ACF as biomarkers of colon cancer by examining the sequential developmental of early lesions in the colon of F344 rats 6 to 28 weeks after azoxymethane treatment. In order to identify early lesions with a morphologic and developmental relationship with nascent tumors as well as lesions not related to tumorigenesis, we classified the developing lesions by surface examination, quantified them and determined their growth, examined them histopathologically, and analyzed them histochemically for altered β-catenin and cyclin D1 expression. In particular, we searched for lesions similar to the recently reported flat dysplastic ACF (ACFMin) in the Min/+ mice and their wild-type littermates (12).

Materials and Methods

Animals and Chemicals. Male F344/Mol rats from Møllegaard Breeding Center Ltd., L1 Skensved, Denmark, weighing 80 g were housed in polysterol cages in a room with 12 hours light/dark cycle and controlled humidity and temperature. The animals were given water and diet ad libitum and the standard diet from B & K Ltd., N. Humberside, United Kingdom. Azoxymethane was from Sigma Chemicals Co., St Louis, MO and was dissolved in 0.9% NaCl.

Experimental Design

Experiment 1. After 1 week of acclimatization, 28 rats received s.c. injections of azoxymethane (15 mg/kg bw/injection) once weekly for 2 weeks. The animals were killed sequentially from weeks 6 to 28 after the last azoxymethane injection to identify, to characterize and score colonic lesions, and to monitor their developmental growth (for experimental details, see Fig. 3A). When all the unsectioned colon preparations were collected, the surface changes were examined retrospectively to identify early lesions, which we called flat ACF, with a morphologic relationship with the final tumors observed in preparations at week 28, the termination of the experiment. Flat ACF, classic elevated ACF, and tumors were scored before representative lesions were dissected and characterized histopathologically (for experimental details, see Table 1).

Experiment 2. For immunohistochemical analyses of the colonic lesions, additional 16 azoxymethane-treated rats were used in order to ensure formalin fixation for no longer than 2 days; the rats were killed sequentially from weeks 6 to 19 after last azoxymethane treatment (for experimental details, see Table 2).

Scoring of Classic Elevated ACF, Flat ACF, and Tumors. The colons were removed, rinsed in ice-cold PBS, slit open longitudinally, and fixed flat between wet (PBS) filter papers for 48 hours in 10% neutral buffered formalin prior to 3 to 5 seconds staining with 0.2% methylene blue.

Table 1. Histopathologic examination of classical elevated ACF, flat ACF, and tumor in the colon of azoxymethane-treated F344 rats

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Surface morphology</th>
<th>Classical elevated ACF</th>
<th>Flat ACF</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 6-14*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia without dysplasia</td>
<td>10/22 (5.5 ± 2.0)†</td>
<td>0/8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>6/22 (4.5 ± 1.5)</td>
<td>0/8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>6/22 (6.3 ± 1.5)</td>
<td>0/8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>0/22</td>
<td>8/8 (10.7 ± 7.7)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Wk 16-19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia without dysplasia</td>
<td>20/33 (5.2 ± 1.8)</td>
<td>0/7</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>11/33 (4.8 ± 2.9)</td>
<td>0/7</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>2/33 (7.5 ± 2.1)</td>
<td>0/7</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>0/33</td>
<td>7/7 (19.9 ± 7.6)</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>Wk 28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia without dysplasia</td>
<td>4/14 (6.5 ± 1.3)</td>
<td>0/1</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>5/14 (5.0 ± 2.4)</td>
<td>0/1</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>5/14 (6.6 ± 2.9)</td>
<td>0/1</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>0/14</td>
<td>1/1 (8)</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Hyperplasia without dysplasia</td>
<td>34/69 (5.4 ± 1.8)</td>
<td>0/16</td>
<td>0/5</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>22/69 (4.7 ± 2.4)</td>
<td>0/16</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>13/69 (6.6 ± 2.1)</td>
<td>0/16</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>0/69</td>
<td>16/16 (14.5 ± 8.6)</td>
<td>5/5</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Experiment 1.

*No. rats (n) used at each time point after azoxymethane treatment: wk 6 (n = 1), wk 10 (n = 2), wk 12 (n = 1), wk 14 (n = 1), wk 17 (n = 2), wk 18 (n = 3), wk 19 (n = 2), and wk 28 (n = 2).

† Mean focal crypt multiplicity ± SD as determined by histological examination in parentheses.
(George T. Gurr Ltd., London, United Kingdom) dissolved in the same formalin solution. Deeply stained crypts were examined by transillumination in an inverse light microscope at least 24 hours after staining. Classic elevated ACF were characterized by their enlarged crypts microscopically elevated from the surrounding epithelium, thickened layer of epithelial cells, increased pericryptal space, and their round or elongated luminal openings. Flat ACF were characterized by their bright blue staining, moderate enlarged or small crypts not elevated from the surrounding epithelium and their compressed round or elongated luminal openings, observable as a streak in the microscope. Because the flat ACF were not observed as elevated structures, their bright blue appearance and compressed pit pattern seen with transillumination were used as criteria for their identification. The size of the lesions was scored as crypt multiplicity (AC/lesion). The crypt multiplicity of lesions with >32 aberrant crypts, which were defined as tumors, was determined by transforming their diameter (mm) to crypt multiplicity. Diameters were scored with an eyepiece graticule. The relationship between tumor diameter in mm (\(d\)) and crypt multiplicity (c) was empirically determined to be \(c = \frac{20}{d^2}\).

### Histopathologic Examination

Areas with mucosal lesions, identified by surface examinations of whole mount colon preparations in the inverse light microscope, were dissected, embedded in paraffin wax, cut in parallel with the mucosal surface, and stained with H&E. A pathologist unaware of the topographical classification of the lesion examined serial sections from different levels of the crypts. Histopathologic classification was based on the following criteria. **Hyperplasia with no dysplasia**: slightly dilated crypts with normal epithelium. **Mild dysplasia**: nuclei elongated, slightly crowded, and pseudostratified, but polarity well preserved, normal or slightly reduced number of goblet cells. **Moderate dysplasia**: nuclei elongated, more crowded, and pseudostratified than in mild dysplasia but polarity partly lost, numerous mitoses, and number of goblet cells markedly reduced or completely lost.

### Immunohistochemistry

Paraffin-embedded formalin-fixed sections were prepared, deparaffinized, and rehydrated in xylene, graded alcohol, and water. Demasking was done in microwave oven for 12 minutes in Tris-EDTA solution (1 mmol/L Trizma base and 0.1 mmol/L EDTA) at pH 9.1. Staining with monoclonal anti \(\beta\)-catenin (Transduction Laboratories, Lexington, KY, cat. no. C19220) at dilution 1:2,500 and counterstaining with hematoxylin was done manually using DAKO EnVision kit and System Peroxidase (DAB). The criteria used for estimation of altered \(\beta\)-catenin staining in lesions compared with surrounding normal tissue was reduced staining at the plasma membrane and increased level in the cytoplasm in addition to the presence of nuclear staining. For cyclin D1, the criterion was the presence of prominent nuclear staining.

### Statistical Analysis

\(\chi^2\) test was used to calculate the statistical difference of proportions between groups. Spearman rank order test was used for correlation analyses. The Mann Whitney rank sum test was used to compare two groups, and one-way ANOVA on ranks was used to compare multiple groups.

### Results

The colons were prepared sequentially from weeks 6 to 28 after the last azoxymethane treatment. By transillumination of the unsectioned methylene blue–stained colon preparations, the surface changes were examined retrospectively in order to identify

### Table 2. Histopathological examination of classical elevated ACF, flat ACF, and tumor in the colon of azoxymethane-treated F344 rats

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Classical elevated ACF</th>
<th>Flat ACF</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta)-Catenin</td>
<td>Cyclin D1</td>
<td>Crypts/focus</td>
</tr>
<tr>
<td>Wk 6-14*</td>
<td>0/25</td>
<td>1/25</td>
<td>4.3 ± 2.5</td>
</tr>
<tr>
<td>Hyperplasia without dysplasia</td>
<td>—</td>
<td>—</td>
<td>3/3</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>0/31</td>
<td>3/31</td>
<td>5.1 ± 2.5</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>0/12</td>
<td>0/12</td>
<td>4.5 ± 1.8</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Wk 16-19</td>
<td>0/15</td>
<td>1/15</td>
<td>6.5 ± 2.4</td>
</tr>
<tr>
<td>Hyperplasia without dysplasia</td>
<td>—</td>
<td>—</td>
<td>5/5</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>0/12</td>
<td>1/15</td>
<td>7.2 ± 2.6</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>0/4</td>
<td>1/4</td>
<td>6.0 ± 2.2</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>—</td>
<td>—</td>
<td>8/8</td>
</tr>
</tbody>
</table>

**Note:** Experiment 2. *No. rats (n) used at each time point after AOM treatment: wk 6 (n = 4), wk 13 (n = 2), wk 16 (n = 2), wk 17 (n = 1), and wk 19 (n = 7).
early lesions with a morphologic relationship with the nascent and developing tumors. Based on surface morphology, all the observed lesions were classified and quantified. Finally, a pathologist unaware of the topographical classification examined lesions histopathologically.

**Classic Elevated ACF and Flat ACF.** The surface examination revealed two types of ACF: classic elevated ACF (Fig. 1A) and flat ACF (Fig. 1C and E). The crypts of flat ACF were distinguished from the flat crypts of the surrounding mucosa by their bright methylene blue staining and their compressed luminal openings, observable as a streak in the microscope. Large flat ACF (Fig. 1E) and tumors (Fig. 1G and I) exhibited characteristic branched or gyrus-like pit patterns of compressed crypts. Large flat ACF (Fig. 1E) and nascent tumors (Fig. 1G), which displayed in principle the same surface morphology, were slightly elevated because of enlargement of the surrounding crypts. This was most prominent in tumors, where the enlarged surrounding crypts seemed to be an integrated part along the margin of the lesion (Fig. 1I).

By histopathologic examination, the lesions were classified as hyperplasia without dysplasia (Figs. 1B and 2A), mild dysplasia (Fig. 2F), moderate dysplasia (Fig. 2H), or severe dysplasia (Figs. 1D, F, H and J and 2D and G). Of all classic elevated ACF examined, 34 of 69 showed hyperplasia without dysplasia, 22 of 69 showed mild dysplasia, and 13 of 69 showed moderate dysplasia (Table 1). There was no statistically significant change of this proportion from week 6 to 14 to week 28, and the mean focal crypt multiplicity did not differ significantly between the categories. None of the classic elevated ACF showed severe dysplasia. In contrast, all the examined lesions initially identified as flat ACF by their surface morphology, showed severe dysplasia (16 of 16).

In total, the flat ACF examined were significantly larger than the elevated classic ACF with hyperplasia and mild dysplasia ($P < 0.001$) but not larger than those with moderate dysplasia.

**Figure 1.** Morphological features of classical elevated ACF, flat ACF, and tumors in the colon of azoxymethane-treated F344 rats. Left, lesions were identified in unsectioned colon preparations briefly stained with methylene blue by transillumination and surface examination in the inverse light microscope (A, C, E, G, and I). Right, histological sections (B, D, F, H, and J) are from the same lesions as seen in the unsectioned colon (left). In cross-sections, classical elevated ACF as identified by surface examination (A) were recognized as lesions without dysplasia (B) or lesions with mild to moderate dysplasia (see Fig. 2H and I). Arrowheads, flat ACF (C and E), which were distinguished from the flat crypts of the surrounding mucosa by their bright methylene blue staining and their pit pattern of compressed crypts, were recognized as lesions with severe dysplasia (D and F), frequently with Paneth cells (arrowhead, D, higher magnification upper right corner; see Fig. 2D). As in flat ACF, surface examination of tumors (lesions with >32 crypts) revealed pit pattern of compressed crypts (arrowheads G and I), and histopathological examination revealed crypts with severe dysplasia, often with Paneth cells (H and J). The dysplastic crypts of tumors were surrounded by enlarged, nondysplastic crypts (I and J) that extended under the lesion (J) from the margin. Magnification: surface images ×100 and histological sections ×190. Diameter of a histologically normal crypt is ~30 μm.
All the tumors showed severe dysplasia (5 of 5). Paneth cells were never observed in classic elevated ACF, but were frequently seen in flat ACF (13 of 16; arrowhead, Figs. 1D and 2G) and in tumors (3 of 5; Fig. 1H and J). The dysplastic crypts of tumors were surrounded by enlarged, hyperplastic crypts without dysplasia (Fig. 1I and J) that extended under the lesion (Fig. 1F) from the margin. Of the five tumors examined, two were adenomas and three were carcinomas.

In an additional experiment, classic elevated ACF, flat ACF, and tumors were examined by immunohistochemistry for cytoplasmic/nuclear overexpression of β-catenin and nuclear overexpression of cyclin D1 (Table 2; Fig. 2). Classic elevated ACF did not show (0 of 99) altered expression of β-catenin (Fig. 2B) compared with normal adjacent crypts where the β-catenin expression was restricted to the membrane of the cell-cell borders. Whereas a small proportion of classic elevated ACF exhibited nuclear overexpression of cyclin D1 (7 of 99, data not shown), the vast majority did not (Fig. 2C). Rare cases of overexpression of cyclin D1 were even seen in histologically normal crypts (data not shown). All flat ACF (8 of 8) and tumors (5 of 5) analyzed, displayed altered expression of both β-catenin, and increased nuclear cyclin D1 expression (Fig. 2E and F). Severe dysplasia and overexpression of β-catenin and cyclin D1 was even observed in one flat ACF constituting one to two crypts at week 6 (data not shown). However, the immunostaining was significant weaker than in larger lesions. Nuclear translocation of β-catenin was more frequent in large flat ACF than in small flat ACF. In total, the flat ACF examined were significantly larger than the cross-section reveals more crypts, probably because of crypt fission deeper in the lesion. Severe dysplasia of a flat lesion; arrowheads. Paneth cells (G), moderate dysplasia from a classical elevated ACF (H), mild dysplasia from a classical elevated ACF (I). Magnification: A-F, ×100; G-I, ×400.

Formation and Growth of Classic Elevated ACF. The number of classic elevated ACF (Fig. 3A) decreased along the time course ($r = -0.77, P < 0.001$), from 180/rat at weeks 6 to 14 to 79/rat at week 28 (Table 3). Their mean crypt multiplicity increased modestly (Fig. 3B), from 3.8 crypt/lesion at weeks 6 to 14 to 5.3 crypts/lesion at week 28 (Table 3; $P < 0.001$).

Formation and Growth of Flat ACF and Tumors. Flat ACF (lesions with crypt multiplicity <32) were observed from week 6 after azoxymethane treatment (Table 3), and their number declined significantly along the time course ($r = -0.42, P = 0.026$). Correspondingly, the number of tumors (lesions with crypt multiplicity >32) increased from 0 at weeks 6 to 14 to 2.5 lesions/animal at week 28 ($r = 0.68, P < 0.001$). From week 6 to the termination at week 28, the numbers of flat ACF + tumors were virtually constant at a level of ~2.5 lesions/animal (Fig. 4A). These data indicate that flat ACF grow fast and that almost all of them had grown to the size of a tumor (lesion with >32 crypts) by week 28. Such a probable developmental interconnection between flat ACF and tumors was also illustrated by the progressive increase of crypt multiplicity observed for these lesions along the time course of tumorigenesis (Fig. 4B). At weeks 6 to 14, before any tumors were observed (Table 3), flat ACF had 2.5-fold higher crypt multiplicity ($P < 0.001$) than classic elevated ACF. The different growth potential of flat ACF and classic elevated ACF was also shown by their size distributions (Fig. 5).

Discussion

By transillumination and surface examination of unsectioned colon preparations of azoxymethane-treated rats, we identified a specific type of ACF, flat ACF that seemed directly associated with tumorigenesis. Flat ACF were characterized by their bright blue

Figure 2. Immunohistochemical analyses of β-catenin and cyclin D1 (brown) of flat ACF and classical elevated ACF in the colon of azoxymethane-treated F344 rats and histological sections of lesions with mild, moderate, or severe dysplasia. Elevated hyperplastic ACF (A), with no altered expression of β-catenin (B) or cyclin D1 (C). Flat dysplastic ACF (D) with altered expression of both β-catenin (E) and cyclin D1 expression (F); this flat lesion had <32 crypts when scored by surface examination, but the cross-section reveals more crypts, probably because of crypt fission deeper in the lesion. Severe dysplasia of a flat lesion; arrowheads. Paneth cells (G), moderate dysplasia from a classical elevated ACF (H), mild dysplasia from a classical elevated ACF (I). Magnification: A-F, ×100; G-I, ×400.
staining, their moderate enlarged or small crypts not elevated from the surrounding epithelium, and their compressed round or elongated luminal openings. Because flat ACF were not observed as elevated structures, their bright blue appearance and compressed pit pattern seen with transillumination were used as criteria for their identification. At a later stage, the crypts surrounding large flat ACF became enlarged, a change that slightly raised the structure; principally, there was no morphologic difference between large flat ACF and nascent tumors. The uniform picture of severe dysplasia with frequent presence of Paneth cells, compressed pit pattern, and Wnt pathway activation (i.e., concomitant overexpression of cytoplasmic/nuclear β-catenin and nuclear cyclin D1) observed by sequential analyses of flat ACF and tumors, indicated a qualitative relationship between these lesions. We also observed quantitative relationships between flat ACF and tumors. Along the time course, the number of flat ACF decreased whereas the number of tumors increased reciprocally keeping the number of flat ACF and tumors constant. Flat ACF and tumors also seemed to have the same large growth rate. Collectively, our qualitative and quantitative data indicated a continuous developmental growth from small flat dysplastic ACF to the stage of a tumor. Apparently, flat ACF and tumors represented the same type of dysplastic lesions at different stages of crypt multiplication. In contrast, classic elevated ACF (1) did not seem to be as clearly related to tumorigenesis. They never possessed severe dysplasia with presence of Paneth cells, compressed crypts, or Wnt pathway activation as seen in flat ACF. In addition, their large number and low crypt multiplicity did not correspond with the low number and fast growth of tumors.

The flat dysplastic ACF that we describe in the present work in azoxymethane-exposed rats seems identical to the fast growing flat dysplastic lesions with altered control of β-catenin (ACF<sub>Min</sub>) that we previously identified by surface examination in the colon of azoxymethane-treated <i>Min</i>+/− mice and in wild-type mice (12) and in untreated <i>Min</i>+/− mice (10). Also, in these animals, we identified two distinct populations of altered crypts. Whereas the fast growing ACF<sub>Min</sub> showed a continuous development from the monocryptal stage to adenoma, the classic elevated ACF, characterized by hyperplasia, normal β-catenin expression, and slow growing crypts, could not directly be linked to tumorigenesis.

The observed flat dysplastic ACF also seem related to the β-catenin-accumulated crypts (BCAC) and the mucin-depleted foci, both described in the colon of azoxymethane-treated rats (18, 21). Whereas the BCAC were only detectable in histologic cross-sections, mucin-depleted foci were detected by high iron diamin Alcian blue staining of the unsectioned colon.

<table>
<thead>
<tr>
<th>Table 3. Number and crypt multiplicity of classical elevated ACF, flat ACF, and tumors in the colon of azoxymethane-treated F344 rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. lesions/rat</strong></td>
</tr>
<tr>
<td>Wk 6-14</td>
</tr>
<tr>
<td>Classical elevated ACF</td>
</tr>
<tr>
<td>Flat ACF</td>
</tr>
<tr>
<td>Tumor</td>
</tr>
</tbody>
</table>

NOTE: Experiment 1. Mean ± SD.
present flat ACF, the BCAC and the mucin-depleted foci were described as histologically more dysplastic than classic elevated ACF. Hence, flat ACF, BCAC as well as mucin-depleted foci seem to be more relevant biomarkers of colon cancer than the classic elevated ACF. They are histopathologically closer to adenomas and carcinomas, their number and crypt multiplicity are correlated with carcinogenesis, and they respond to promotional (22) and chemopreventive (11, 21, 23) agents in parallel with the tumors. However, in the limited number of studies to date, the numbers detected of ACF, BCAC and mucin-depleted foci, ~3/colon, ~20 to 30/colon, and ~8/colon, respectively, differed significantly. Additional studies are therefore needed to clarify the relationship between these lesions and to clarify whether the methods of detection may influence the score. The compressed pit pattern and flat feature of the flat ACF observed in rat colon also seem to be related to the so-called flat adenoma detected in the human colon (24).

The characteristic phenotype of flat ACF is apparently closely associated with aberrant activation of the Wnt signaling pathway. Although we did not examine the genotypes of the flat ACF in the present study, frequent β-catenin gene mutations have been reported in BCAC (25) in the rat colon and in colonic tumors from rats and mice exposed to azoxymethane (15, 26).

Activated Wnt signaling pathway may explain the immature (dysplastic) appearance of crypt cells constituting flat ACF, because this signal is essential for the maintenance of the proliferative compartments of the intestine during embryogenesis (27). The flat appearance of these dysplastic lesions could be a result of disrupted Apc control of the cell cycle (28) and cell

Figure 4. Sequential development of flat ACF and tumors (lesions with severe dysplasia) in the colon of F344 rats 6 to 28 weeks after azoxymethane treatment. Experiment 1, A, number of flat ACF (●) and tumors (○) in each individual rat. B, crypt multiplicity of all the (●) flat ACF and tumors (○); point, one lesion.

Figure 5. Size distribution of classical elevated ACF, flat ACF, and tumors in the colon of F344 rats at weeks 6 to 14, 16 to 19 and 28 after azoxymethane treatment. Experiment 1, A, classical elevated ACF. B, flat ACF. C, tumors. Weeks 6 to 14 (---), weeks 16 to 19 (⋯⋯) and week 28 (- - -). The size is expressed as crypt multiplicity (no. crypts/lesion), and each size class unit represents a successively doubling of crypts (1-2, 3-4, 5-8, 9-16, 17-32, etc.).
anchoring (29), as well as disrupted Apc-driven migration (30) and apoptosis (31).

Classic elevated ACF exhibited hyperplasia without dysplasia, mild dysplasia, or moderate dysplasia. It is noteworthy that they never showed severe dysplasia with the presence of Paneth cells or Wnt pathway activation. A few classic elevated ACF as well as normal crypts showed overexpression of cyclin D1, indicating that this gene may be activated independently of β-catenin signaling. Classic elevated ACF, as we observe them, do not seem to be directly related to tumorigenesis, although 50% of them possessed mild to moderate dysplasia. This accords with the fact that classic elevated ACF with moderate dysplasia could be induced by azoxymethane in mice resistant to colonic carcinogenesis (32). Initially there were ~100 times more classic elevated ACF than flat ACF. However, their number regressed by 50% from weeks 6 to 28.

Although our results do not indicate a direct morphologic link between the classic elevated ACF and tumors, it cannot be excluded that some classic elevated ACF represent an earlier stage than flat ACF, particularly at the monocryptal stage. Neither can it be excluded that some large classic elevated ACF might acquire additional mutations that may transform them into lesions with severe dysplasia and aberrant Wnt activation. Although we did not observe intermediate lesions indicative of such a transformation in our study, they could in theory exist, particularly in animal models using long-term multiple carcinogen exposure and in human colon carcinogenesis. In human ACF, epigenetic changes that would make them particularly sensitive to malignant changes might be initiated by mutations in APC or β-catenin have been detected (33). Moreover, it might well be that those lesions previously recognized as late stage classic elevated ACF with severe dysplasia in rodent and human colon carcinogenesis (5, 6, 19, 34–37) are identical with the advanced stages of flat ACF described by us. In particular, this might be the case with large human dysplastic ACF that have altered expression of β-catenin (19).

In conclusion, in the colon of rats exposed twice to azoxymethane, we identified two distinct populations of altered crypts: flat ACF and classic elevated ACF. The lesions could be recognized by transillumination and surface examination in the inverse microscope of unsectioned colon preparations briefly stained with methylene blue. Flat ACF displayed a continuous development from early stages to adenoma with fast crypt multiplication and aberrant activation of the Wnt signaling pathway. This is consistent with the observation that inactivation of APC is sufficient for the growth of early colorectal adenomas. In contrast, classic elevated ACF do not seem to be as closely related to tumorigenesis.

References


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References


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Flat Dysplastic Aberrant Crypt Foci Are Related to Tumorigenesis in the Colon of Azoxymethane-Treated Rat

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