Prevalent Location of Flat Dysplastic Aberrant Crypt Foci near Lymphoid Follicles in the Colon of Azoxymethane-treated Rats

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Abstract. In the colon of F344 rats treated with 2 x 15 mg/kg body weight of azoxymethane (AOM), the density (number of lesions/cm²/rat) of flat aberrant crypt foci (ACF) was 13-fold higher (p<0.05) in the surface area of mucosa immediately adjacent to lymphoid follicles compared with the density of these lesions in the rest of the mucosa. A similar prevalent location near lymphoid follicles was observed for tumours, but not for the classic elevated ACF. The lymphoid follicle-associated flat ACF had the same characteristics as those located in the rest of the mucosa: i.e. severe dysplasia and Wnt pathway stimulation.

Aberrant crypt foci (ACF) are described as putative preneoplastic lesions in the colon of carcinogen-treated rodents (1) and in human colorectal cancer. Because ACF protrude towards the lumen, they are easily scored by light microscopic surface examination of formalin-fixed whole-mount colon preparations stained with methylene blue, and they are frequently used as a short-term bioassay to evaluate the role of nutritional components and chemopreventive agents at an early stage of colon carcinogenesis. Recently, flat ACF with severe dysplasia were discovered, in addition to the classic protruding ACF in rats treated with azoxymethane (AOM) (2). Apparently, there was a continuous developmental growth from small flat dysplastic ACF to the stage of a tumour and classic elevated ACF were not as closely related to tumorigenesis. Flat ACF were also observed in Min mice, a murine FAP model, their wild-type littermates treated with AOM (3), as well as in A/J mice treated with AOM (4). Wnt pathway activation, observed as overexpression of β-catenin, cyclin D1, was characteristic of flat ACF and tumours but not for classic elevated ACF (2-4).

Clusters of lymphoid follicles are normal features in the distal, mid and proximal colon of rats, interspersed by solitary follicles (5). There were no significant differences between saline-treated and 1,2-dimethylhydrazine (DMH)-treated rats regarding the size, cellularity and number of lymphoid follicles per rat (6). In a large number of rodent studies, a close spatial association between DMH/AOM-induced colonic tumours and pre-existing colonic lymphoid follicles was demonstrated (6-14). In humans, a close relationship between adenomas and lymphoid follicles was also observed (15).

Even though lymphoid follicles represent a limited area for histological or surface examination, only a few reports have described potential early precursor lesions in these structures (12-17). In rodents, a lack of correlation between the spatial distribution of ACF and the occurrence of tumours, which were predominantly formed in association with lymphoid follicles, was reported (12, 14). However, the same group described microscopic, endophytic adenocarcinomas within the lymphoid follicles (13). Human ACF also displayed a coincidence with lymphoid follicles that were 2.5 (hyperplastic ACF) to 8 times (dysplastic ACF) higher than expected (16).

In the present work, our aims were to: (i) examine whether flat ACF and classic elevated ACF accumulate in the area immediately adjacent to lymphoid follicles in the colon of AOM-treated rats; (ii) examine whether early lesions, located in the area near lymphoid follicles, are histologically different from similar lesions located in the rest of the mucosa.

Materials and Methods

After one week of acclimatisation, male F344/mol rats (Møllegaard Breeding Center Ltd., L. Skensved, Denmark), weighing 80 g, were injected s.c. with azoxymethane (AOM, Sigma Chemicals Company, St. Louis, MO, USA) dissolved in 0.9% NaCl once weekly for 2 weeks (15 mg/kg body weight (bw)/ injection).
Table I. Distribution (number/rat) and density (number/cm²/rat) of colonic lesions in the surface area of mucosa immediately adjacent to lymphoid follicles or the surface area of the rest of the mucosa of AOM-treated F344 rats. Experiment 1.

<table>
<thead>
<tr>
<th>Week 8 (n=6)</th>
<th></th>
<th>Week 25 (n=8)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymphoid follicle</strong></td>
<td><strong>Rest of the mucosa</strong></td>
<td><strong>Lymphoid follicle</strong></td>
<td><strong>Rest of the mucosa</strong></td>
</tr>
<tr>
<td><strong>Area (mm²)</strong></td>
<td>62±4</td>
<td>1854±128</td>
<td>74±15</td>
</tr>
<tr>
<td>Classic elevated ACF/rat</td>
<td>8.0±3.9</td>
<td>277±31</td>
<td>1.0±1.1</td>
</tr>
<tr>
<td>Classic elevated ACF/cm²/rat</td>
<td>12.86±6.40</td>
<td>14.90±1.30</td>
<td>1.23±1.32</td>
</tr>
<tr>
<td>Flat ACF/rat</td>
<td>0</td>
<td>1.7±1.4</td>
<td>0</td>
</tr>
<tr>
<td>Flat ACF/cm²/rat</td>
<td>0</td>
<td>0.09±0.07</td>
<td>p=0.023</td>
</tr>
</tbody>
</table>

The surface area immediately adjacent to lymphoid follicles is defined as the surface area of mucosa less than 600 μm from the margin of the follicle. n.s. = not significant.

The colons were removed, rinsed in ice-cold PBS, slit open longitudinally and fixed flat between wet (PBS) filter papers for 48 h in 10% neutral buffered formalin prior to 3-5 sec staining with 0.2% methylene blue (George T. Gurr Ltd., UK) dissolved in the same formalin solution. Deeply stained crypts were examined by transillumination in an inverse light microscope at least 24 h after staining. Classic elevated ACF were characterised by their enlarged crypts, microscopically elevated from the surrounding epithelium, a thickened layer of epithelial cells, increased pericryptal space and their round or elongated luminal openings. Flat ACF were characterised by their bright blue staining, moderately 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. The lesions were classified histopathologically as previously described (2). Staining with monoclonal anti-β-catenin (Transduction Laboratories, Lexington, KY, cat. no. C19220) and monoclonal anti-cyclin D1 (Zymed Laboratories Inc., San Francisco, CA, cat. No. ZS18-0220) was performed as previously described (2).

In experiment 1, the rats were sacrificed at week 8 (n=6) after the last AOM injection to determine the distribution of classic elevated ACF and flat ACF in the mucosa immediately adjacent to lymphoid follicles and in the rest of the mucosa and at week 25 (n=8) to determine the similar distribution of tumours. In experiment 2, AOM-treated rats were sacrificed at weeks 16 (n=4), 18 (n=4) and 19 (n=4) to determine the distribution of the flat ACF and tumours. In order to ensure formalin fixation for no longer than 2 days as required for immunohistochemical analyses, additional rats (experiment 3) were sacrificed at weeks 17 (n=3) and 19 (n=3) after the last AOM treatment.

A one-sample t-test was used to compare the density of lesions linked to lymphoid follicle and the remaining surrounding mucosa.

Results

Whereas the total colonic surface area increased significantly with age, the proportion of surface area of the mucosa immediately adjacent to the lymphoid follicles to the area of the rest of the mucosa (~1/30) did not change (Table I). At week 8 after AOM treatment, no flat ACF were observed in the area immediately adjacent to the lymphoid follicles and a few were observed in the rest of the mucosa (Table I). Even though the vast majority of classic elevated ACF (277/rat) were located outside the area immediately adjacent to lymphoid follicles, the density of these lesions was similar in the two locations. At week 25, the density of tumours in the area immediately adjacent to the lymphoid follicles was significantly higher than the density of tumours in the rest of the mucosa (p=0.039).

In the intermediate phase of tumorigenesis (weeks 16-19), flat ACF were detected in the mucosa immediately adjacent to the lymphoid follicles and the density of flat ACF in these areas was significantly higher than in the rest of the mucosa (p=0.024) (Table II). A similar pattern was seen for the tumours (p=0.049).

The histopathological and immunohistochemical characteristics of the flat ACF and classic ACF located in the area immediately adjacent to lymphoid follicles were
Figure 1. Morphological, histopathological and immunohistochemical characteristics of classic elevated ACF and flat ACF located in the mucosal surface area immediately adjacent to lymphoid follicles in the colon of AOM-treated F344 rats. The lesions were identified by surface examination of unsectioned colonic preparations in the light microscope (A). Arrows indicate the connection between the surface image of a lesion and its cross-section in the columns below. Two classic elevated ACF, show hyperplasia with no dysplasia (C and D) and no overexpression of β-catenin (F and G) or cyclin D1 (I and J). The gyrus-like pit pattern of compressed crypt openings of a flat ACF, as observed by surface examination (B), is recognised in the H&E section (E), which also shows a lesion with severe dysplasia. This flat ACF also exhibits overexpression of β-catenin (H) and cyclin D1 (K). Magnification: (A) 50x, (B-K) 100x.
similar to those reported for the same lesions located in the rest of the mucosa (2). The classic ACF displayed hyperplasia with no dysplasia (Figure 1 C and D), mild dysplasia or moderate dysplasia, but no (0/23) severe dysplasia. In contrast, all the flat ACF (7/7) displayed severe dysplasia (Figure 1 E). Whereas the classic elevated ACF (0/23) showed no concomitant overexpression of β-catenin (Figure 1 F and G) or cyclin D1 (Figure 1 I and J), biomarkers of Wnt pathway activation, the flat ACF (4/4) displayed overexpression of these proteins in all cases (Figure 1 H and K), as in the case of the two tumours (2/2) located adjacent to lymphoid follicles (data not shown).

Discussion

In this study, the significance of lymphoid follicles in rodent colon carcinogenesis was confirmed and additional support to our hypothesis that flat ACF, but not classic elevated ACF, represent early lesions in tumorigenesis (2, 3). In a number of previous studies, a close spatial association between carcinogen-induced colonic tumours and the pre-existing lymphoid follicles was observed (6-14). The present data expand this picture, demonstrating the prevalent location of flat ACF near lymphoid follicles. We observed an increased density of flat ACF in the surface area of mucosa immediately adjacent to the lymphoid follicles compared with the density of these lesions in the rest of the mucosa. In addition, previous results demonstrating a similar accumulation of tumours were confirmed. Furthermore, the lack of co-association between classic elevated ACF and lymphoid follicles is in agreement with previous reports (12, 14).

The flat ACF, located immediately adjacent to lymphoid follicles, may be directly related to the microscopically endophytic adenocarcinomas within the lymphoid follicles described by Hardman and Cameron (12). They also seem related to the human dysplastic ACF, which displayed a coincidence with lymphoid follicles that were eight times higher than expected (16).

The enhanced density of the flat ACF immediately adjacent to the lymphoid follicles, observed at week 16-19 was not consistent with the absence of these lesions in the same area at week 8, particularly because flat ACF were observed in the rest of the mucosa at this early stage. This indicates that the surface morphology of small flat ACF at the early stage of carcinogenesis probably do not differ significantly from the surface morphology of normal crypts near the lymphoid follicles, which frequently display atypical changes and constitute less differentiated cells than the normal crypts further away from the lymphoid follicles (6, 8, 9, 18). However, at weeks 16-19, the difference between lymphoid follicle-associated flat ACF and other normal crypts immediately adjacent to the lymphoid follicles was obviously more prominent, and the flat ACF could easily be identified.

The flat ACF located in the area immediately adjacent to lymphoid follicles had the same characteristics as those located in the rest of the mucosa. The flat lesions exhibited crypts with severe dysplasia, compressed crypt openings and Wnt pathway activation, observed as concomitant overexpression of nuclear/cytoplasmic β-catenin and nuclear cyclin D1, features also characteristic of tumours (2, 3).

The observed prevalent location of flat ACF, or tumours near lymphoid follicles, indicates that those crypts adjacent to lymphoid follicles are more susceptible to mutational and initiating events induced by the carcinogen than are more distant crypts. The increased carcinogenesis in crypts bordering the lymphoid follicles could be due to the enhanced cell proliferation observed in these crypts (13, 19). In general, it is proposed that increased cell proliferation is promotional to carcinogenesis in the colon. It may well be that cells within the lymphoid follicle produce paracrine factors that cause enhanced mitotic activity in the immediately adjacent crypts. Immunohistochemical findings indicate that TGFα may be such a stimulatory and promotional factor for these crypts (13).

In conclusion, the density of flat ACF, or tumours, but not of classical ACF, was higher in the surface area of mucosa immediately adjacent to lymphoid follicles than in the remaining area of the mucosa.

Acknowledgements

This work was supported by The Research Council of Norway and the Norwegian Cancer Society.

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Received February 2, 2006
Accepted March 16, 2006


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