Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis

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Crypt foci with absent or scant mucous production (mucin-depleted foci, MDF) were recently described by our group in the colon of azoxymethane (AOM)-treated rats. Since MDF are dysplastic and easy to quantify, we think that MDF are pre-neoplastic lesions that could be used as biomarkers for carcinogenesis. To test this hypothesis, we studied MDF in azoxymethane (AOM)-initiated rats treated with cholic acid (CHA), a promoter of colon carcinogenesis or with piroxicam (PXC), a colon cancer-inhibiting drug. Aberrant crypt foci (ACF) were determined as well. F344 male rats were treated with AOM (15 mg/kg × 2, s.c.) and then divided into: controls, which were fed AIN76 diet; CHA group, which was fed AIN76 diet containing CHA 0.5% w/w; PXC group, which was fed AIN76 diet containing PXC 0.02% w/w. Ten weeks after the first dose of AOM, the total number of MDF was significantly increased in rats treated with AOM (P < 0.05) and drastically reduced (P < 0.01) in rats treated with PXC (MDF/colon were 6.10±1.26, 10.59±1.96 and 1.31±0.21 in controls, CHA and PXC groups, respectively, means±SE). The multiplicity of MDF was also increased in CHA-treated rats. On the contrary, ACF multiplicity was significantly decreased by CHA. In PXC-treated rats there were fewer ACF with lower multiplicity. The effect of PXC was also investigated 15 weeks after the first AOM dose and the results showed that the total number of MDF in the PXC group was significantly lower than in controls. The number of ‘large’ MDF, formed by 12 or more crypts, was also reduced (P < 0.01) by PXC (‘large’ MDF were 1.7±0.5 and 0.4±0.2 in control and PXC groups, respectively). Since CHA promotes and PXC reduces colon cancer, MDF are correlated with carcinogenesis and can be proposed as endpoints to study the modulation of colon carcinogenesis in short-term experiments.

Introduction

Pre-neoplastic lesions with various degrees of dysplasia represent an obligatory step in colon carcinogenesis and much effort has been dedicated to their identification and characterization in humans and experimental animals (1–6). In fact, since colon carcinogenesis is a long process, taking months to develop in rodents, pre-neoplastic lesions such as aberrant crypt foci (ACF), which occur ~30 days after carcinogen administration, or even earlier, have been extensively used as an endpoint in short-term carcinogenesis and chemoprevention studies (7–9). However, some studies report a lack of correlation between ACF induction and tumour development (10–12), thus challenging the use of ACF as biomarkers for colon carcinogenesis.

While other pre-neoplastic lesions such as β-catenin accumulated crypts (BCAC) have been described recently in carcinogen-treated rodents and proposed as additional biomarkers for colon carcinogenesis (5,13,14), their identification, based on immunohistochemical methods, is problematic in unsectioned colons.

Recently, we identified new lesions in the colon of rats treated with azoxymethane (AOM), formed by crypts characterized by the absence or scant production of mucus (mucin-depleted foci, MDF) (12). MDF are easy to quantify in the entire unsectioned colon and show clear characteristics of dysplasia in histological sections (12). In a study in which prebiotics and probiotics prevented colon cancer development (12,15), we demonstrated that MDF are correlated with carcinogenesis and we suggested that MDF are pre-neoplastic lesions that can be used as a biomarker in colon carcinogenesis (12).

If this hypothesis is correct, MDF should increase with treatments that promote colon cancer and, on the contrary, should be reduced by chemopreventive agents. Accordingly, we thought it of interest to evaluate MDF in AOM-induced rats treated with cholic acid (CHA), a known promoter of colon cancer (16–18) or in rats treated with piroxicam (PCX), a non-steroidal anti-inflammatory drug, which inhibits colon carcinogenesis (7,19,20). MDF were determined at different times after carcinogen administration. We also determined ACF in these animals to compare the results obtained by enumerating these two purported pre-neoplastic lesions.

Materials and methods

AOM, CHA and PCX were purchased from Sigma (Milan, Italy). Dietary components for the preparation of the AIN76 diet were purchased from Piccioni (Gessate, Milan, Italy).

Animals and treatments

We used 4- to 5-week-old, male F344 rats (Nossan, Correzzana, Milan, Italy). The animals were housed according to the European Union Regulations on the Care and Use of Laboratory Animals, as reported previously (21). After their arrival from the supplier, animals (n = 45) were quarantined for 1 week, during which they were fed the AIN 76 diet. Rats were then treated s.c. with two injections (1 week apart) of AOM (15 mg/kg, total dose 30 mg/kg). One week after the last injection of AOM, the rats were randomly allocated to the different dietary treatments: controls (20 rats/group) were fed AIN76 diet; the CHA group (eight rats) was fed AIN76 diet containing 0.5% CHA (w/w); the PCX (17 rats) group was fed AIN76 diet containing 0.02% PCX w/w. Diets containing CHA or PCX were prepared every week, kept refrigerated and fed ad libitum to rats. Control rats were fed ad libitum as well.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCAC, β-catenin accumulated crypts; CHA, cholic acid; MB, methylene blue; MDF, mucin-depleted foci; PXC, piroxicam.
Ten weeks after the first AOM injection, rats were killed by CO₂ asphyxiation and ACF and MDF were determined as described below. An additional group of controls and PXC-treated rats were killed 15 weeks after the first injection of AOM to determine ACF and MDF also at this time point.

**Determination of ACF and MDF**

At death, the colon was removed and pinned flat on a polystyrene board to reduce any folding of the colon mucosa, which interferes with a good visualization of the crypts after the high-iron diamine Alcian blue staining (see below). The polystyrene board was dipped in formalin to fix the colon. ACF were then determined according to Bird (2) by staining colons with methylene blue (MB). We also calculated the number of 'large' ACF, a parameter often used to predict carcinogenesis outcome (7,22,23). 'Large ACF' were defined with two different criteria (22,23): as ACF with a multiplicity (i.e. the number of crypts forming each focus) equal to or higher than 4 crypts/ACF (23) or as ACF being of such a multiplicity that there was the same total number of large ACF in the control group as the number of animals in the group (22). Using this last definition, in ACF determined 10 weeks after the first injection of AOM, 'large' lesions have a multiplicity $\geq$10 crypts, while in those harvested 15 weeks after the first dose of AOM, 'large' lesions are $\geq$12 crypts. After ACF determination, MB-stained colons were kept in formalin and then processed with the high-iron diamine Alcian blue staining (HID-AB) to visualize MDF as described (12,24). The HID-AB-stained colons were scored at the microscope (Optiphot-2, Nikon, NIKON, Japan) (40× magnification) and MDF were identified as focal lesions characterized by the absence or very limited production of mucins (Figure 1). Besides this defect in mucin production, MDF can be recognized since they are focal lesions and are formed by crypts with a lumen, which is often distorted when compared with normal surrounding crypts. Elevation of the lesion above the surface of the colon, and a multiplicity (i.e. the number of crypts forming each focus) of $\geq$3 crypts, are also frequent features of MDF (Figure 1). We also determined the number of 'large' MDF defined with the same criteria specified above for ACF. The colons were coded and scored independently by two observers. The correlation coefficient between scores of two observers on a set of 26 colon samples was 0.95 ($P < 0.001$) for the number of MDF/colon and 0.92 ($P < 0.001$) for the multiplicity of MDF (number of crypts forming each focus).

The size of MDF was determined with a grid placed on the ocular of the microscope, calculating the area occupied by an MDF observed at 100× magnification.

**Statistical analysis**

Data obtained from individual rats in the different groups were analyzed with one-way ANOVA by calculating the contrasts between means using the Duncan’s method for multiple comparisons. Correlation between MDF scores of the two observers was determined with a simple regression model calculating the correlation coefficient and probability values. Calculations were performed using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA). Differences were considered statistically significant when $P$ was $<0.05$.

**Results**

**Body weights**

CHA-treated rats had a significantly lower body weight, compared with controls (Figure 2). This effect was evident as early as 1 week after CHA treatment and persisted for the duration of the experiment with CHA. PXC did not affect body weight (Figure 2).

**ACF and MDF determination 10 weeks after AOM**

Ten weeks after the first AOM injection, rats were killed and ACF determined in MB-stained colon. The number of ACF/colon (Table I) was similar in controls and CHA-treated rats, whereas in PXC-treated rats we observed a significant ($P < 0.01$) reduction of ACF. The multiplicity of ACF was lower in CHA and PXC groups when compared with controls (Table I). We also calculated the number of 'large' ACF, a parameter often used to predict carcinogenic outcome using the two criteria described in the Materials and methods section (7,22,23). The results of this determination (Table II) showed that the number of ACF with a multiplicity $\geq$4 crypts was significantly lower in CHA and PXC groups relative to controls; similarly, the number of ACF formed by $\geq$10 crypts was lower in CHA and PXC groups, although this difference did not attain statistical significance (Table II).

MB-stained colons were then re-stained with HID-AB to determine MDF. Contrary to what we observed with ACF, the number of MDF/colon in rats treated with the cancer
promoter CHA was significantly higher than in controls (Table I). The number of MDF/colon in rats treated with PXC was significantly lower than in controls and in this group the average number of MDF was as low as 1.3/rat while in control rats there were ~6 MDF/colon (see Table I).

The multiplicity of MDF was significantly higher in the CHA-treated group than in controls, while there was no difference in the PXC group (Table I). We also measured the average size of MDF; the results indicated that MDF were significantly larger ($P < 0.01$) in the CHA group than in controls; the PXC group was similar to controls [MDF size was 3.71 ± 0.01, 7.51 ± 0.02, 4.29 ± 0.01 (× 10^-2) mm² in controls, CHA, PXC groups, respectively; means ± SE].

We also determined the number of ‘large’ MDF and saw that the number of ‘large’ MDF was significantly higher in rats treated with CHA than in controls (Table II). In rats treated with PXC, the number of MDF > 4 crypts was significantly lower than in controls. Similar results were obtained when defining ‘large’ MDF as lesions formed by > 10 crypts, but in this case the results did not attain statistical significance (Table II).

**ACF and MDF determination 15 weeks after AOM**

An additional group of controls and PXC-treated rats were killed 15 weeks after the first AOM treatment. As observed at the previous time point, the number, the multiplicity of ACF and the number of ‘large ACF’ were significantly lower in the PXC group than in controls (Tables I and II).

### Table I. Number of total ACF and MDF/colon and their multiplicity (number of crypts/focus) at different times after the first injection of AOM in control rats or in rats treated with CHA (0.5% w/w in the diet) or PXC (0.02% w/w in the diet)

<table>
<thead>
<tr>
<th>Time after AOM</th>
<th>10 weeks</th>
<th>15 weeks</th>
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<tbody>
<tr>
<td></td>
<td>ACF</td>
<td>MDF</td>
</tr>
<tr>
<td></td>
<td>Total ACF/colon</td>
<td>Crypts/ACF</td>
</tr>
<tr>
<td>Controls</td>
<td>210 ± 17 (10)</td>
<td>2.96 ± 0.03 (10)</td>
</tr>
<tr>
<td>CHA</td>
<td>182 ± 16 (8)</td>
<td>2.56 ± 0.08 (8)</td>
</tr>
<tr>
<td>PXC</td>
<td>62 ± 10 (8)</td>
<td>2.50 ± 0.04 (8)</td>
</tr>
</tbody>
</table>

**Values are means ± SE, numbers in parentheses are the number of rats/group.**

<table>
<thead>
<tr>
<th>Time after AOM</th>
<th>10 weeks</th>
<th>15 weeks</th>
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<tbody>
<tr>
<td></td>
<td>‘large’ ACF</td>
<td>‘large’ MDF</td>
</tr>
<tr>
<td></td>
<td>≥4</td>
<td>≥10</td>
</tr>
<tr>
<td>Controls</td>
<td>61.5 ± 15.3 (10)</td>
<td>1.2 ± 0.7 (10)</td>
</tr>
<tr>
<td>CHA</td>
<td>37.4 ± 6.1 (8)</td>
<td>0.9 ± 0.3 (8)</td>
</tr>
<tr>
<td>PXC</td>
<td>12.8 ± 3.3 (8)</td>
<td>0.4 ± 0.3 (8)</td>
</tr>
</tbody>
</table>

**Values are means ± SE, numbers in parentheses are the number of rats/group.**

**‘Large’ ACF or MDF were defined as: lesions with a multiplicity ≥4 or ≥10 as lesions being of such a multiplicity that at least one ‘large’ lesion/rat is present in controls. Using this last definition, in ACF and MDF determined 10 weeks after the first AOM injection, ‘large’ lesions have a multiplicity ≥10 crypts, while in those harvested 15 weeks after the last injection of AOM, ‘large’ lesions are ≥12 crypts.**

The number of MDF in the PXC group was also considerably lower than controls (Table I); while MDF multiplicity was slightly reduced but this effect did not attain statistical significance (Table I). The number of ‘large’ MDF was also significantly lower in the PXC group than in controls (Table II).

### Discussion

MDF, newly identified lesions in AOM-treated rats (12), are candidate biomarkers for colon carcinogenesis. They are formed in response to known promoters of colon carcinogenesis or to chemopreventive agents.

The present data demonstrate that the number of MDF, their multiplicity and the number of ‘large’ MDF are significantly increased by CHA, a known promoter of colon carcinogenesis in different experimental models (16–18). The number of ‘large’ MDF, defined using the same criteria as ‘large’ ACF (22,23), is also increased in CHA-treated rats, further supporting the idea that MDF can predict cancer. Recently, Hirose et al. (13) demonstrated that CHA also increases the multiplicity, but not the number, of BCAC, purported pre-neoplastic lesions in rat colon (5). We do not know at the present if BCAC and MDF are related lesions; in fact, while MDF can be counted in the entire unsectioned colon, BCAC are identified in histological sections with immunohistochemical techniques.
However, it is interesting to note that BCAC, like MDF, have a low production of mucus (5).

CHA administered at 0.5% in the diet significantly decreases body weight, as documented by others in carcinogen-treated rats (13,18); lower concentrations of CHA (0.2% in the diet) have also been reported to decrease body weight in rats not treated with AOM (10). Since caloric restriction depresses chemical carcinogenesis (7), the lower body weight observed in CHA-treated rats might have hampered the enhancing effect of CHA on ACF and MDF. Actually, MDF were higher in the group with lower body weight and lower in a group with body weight similar to controls (PXC group), therefore, the variation in body weight did not affect the results obtained, at least in the case of MDF.

In the present study we also tested the effect of PXC, a non-steroidal anti-inflammatory drug on MDF. PXC is repeatedly reported to decrease AOM-induced colon cancer (19,20) and have also been reported to decrease body weight in rats not treated with AOM (10). Since caloric restriction depresses chemical carcinogenesis (7), the lower body weight observed in CHA-treated rats might have hampered the enhancing effect of CHA on ACF and MDF. Actually, MDF were higher in the group with lower body weight and lower in a group with body weight similar to controls (PXC group), therefore, the variation in body weight did not affect the results obtained, at least in the case of MDF.

In this study we also compared ACF with MDF. Up to now, ACF are considered the ‘gold standard’ of colon carcinogenesis biomarkers (7) and ACF determination is widely used to identify potential chemopreventive agents (7,9). Our study demonstrates that in PXC-treated rats, ACF determination is correlated with MDF, showing that in this case both lesions predict carcinogenesis. On the contrary, CHA, administered during the promotion phase of carcinogenesis, decreased ACF multiplicity and the number of ‘large’ ACF.

Lack of agreement between ACF and carcinogenesis in CHA-treated rats has been reported before (10,13) and different results have been obtained with different experimental protocols and by varying the timing of CHA administration (26). In fact, CHA increased the number of ACF (10,26,27) and increased their multiplicity in carcinogen-initiated rats (10). It has also been suggested that the tumour-enhancing effect of CHA is exerted only during initiation, through the induction of lesions with an enhancing growth phenotype in the very early stages of ACF formation (26). Whatever the effect of CHA on ACF might be, since the protocol we used is associated with tumour induction (AOM x 2, followed by 0.5% CHA) (18), it is clear that ACF are not correlated with carcinogenesis, at least in these experimental conditions.

This lack of correlation has been reported with other substances (11,12) and may be due to the heterogeneity of the multitude of ACF induced by AOM. Accordingly, it has been suggested that only a subgroup of ACF, those with dysplastic characteristics, progress to cancer, while the rest of non-dysplastic ACF are not directly related to tumour formation (14,28,29). Therefore, only if a substance has similar effects on both types of ACF, will the results be correlated with carcinogenesis. Theoretically, if the substance has different effects on dysplastic and non-dysplastic ACF, the results of the test, reflecting the entire population of ACF, will not predict carcinogenesis. Accordingly, the ability of MDF to predict carcinogenesis that we observed here and in a previous chemoprevention study (12) might be related to the fact that MDF are less heterogeneous than ACF, a hypothesis that should be tested by further characterization of these lesions. It is also important to note that 30 mg/kg of AOM induce ~200 ACF/colon, whereas the number of MDF is much lower (<10/colon) and in the same order of magnitude of tumours (12); this fact, which should be further confirmed by other studies, could explain why MDF results may predict carcinogenesis better than ACF.

In conclusion, we found that MDF are more numerous and larger in CHA-treated rats, whereas MDF formation is depressed in those treated with PXC. Since CHA is known to promote colon cancer and PXC to inhibit it, our results demonstrate that MDF are correlated with carcinogenesis and can be proposed as biomarkers in short-term colon carcinogenesis experiments.

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