Early Oncofetal Antigenic Modifications during Rat Colonic Carcinogenesis

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ABSTRACT

M1 antigens, associated with adult rat surface gastric epithelium and which are present in fetal but not adult distal colon, were investigated in this colon mucosa during carcinogenesis. Fifty Wistar rats were given s.c. injections of 1,2-dimethylhydrazine for 28 weeks. Using an immunohistochemical method, M1 antigens associated with goblet cells were shown to be present after 2 weeks of 1,2-dimethylhydrazine treatment in histologically normal mucosa and then in 78% of mucinous hyperplasia and polyoid-like glands, in 54% of hyposecreting glands, and in 58% of dysplasias, Grades 1 and 2, in two of 12 dysplasias, Grade 3, and in five of five transitional mucosas adjacent to carcinoma. The production of sialomucins associated with M1 antigens was often seen in the same histological lesion, although not always associated in the same goblet cells. The number of these histological lesions as well as the production of sialomucins associated with M1 antigens increased with the number of injections. Thus, these antigenic changes of an oncofetal nature can be regarded as early modifications of goblet cell differentiation in colonic mucosa subjected to chemical carcinogen.

INTRODUCTION

Changes accompanying carcinogenesis have been investigated for some time. Using histochemical methods, modifications in the mucins produced by goblet cells have been described in human (21) as well as in rat (9) distal colonic mucosa; a predominant production of sialomucins was observed, in association with the colonic cancer process, in apparently normal mucosa adjacent to the tumor and in patches at various distances (11). More recently, using another approach, we found that antigens associated with mucous cells of surface gastric epithelium (M1 antigens) were produced in 29% of human colonic adenocarcinomas (4) and sometimes detected in the transitional mucosa (2); these antigens were also present in fetal colonic mucosa (2). Are these oncofetal-type changes present before tumor appearance? We have tried to answer this question by studying early modifications in colonic mucosa of rats treated with DMH. In this model, most of the histological lesions are comparable to those described in human colorectal cancer (23). Moreover, the immunohistochemical method is possible because human and rat gastric M1 antigens share common antigenic specificity (7). Thus, using this new approach, we have considered whether M1 antigens may be regarded as good markers in the early stages of malignant transformation; we have also compared these changes with those described previously using histochemistry.

MATERIALS AND METHODS

Animals

Sixty-seven 6-week-old female Wistar rats at the beginning of the experiment were used and fed a standard diet provided by Pietrement, Provins, France.

Animals aged 1, 2, 5, and 10 days were also used. Rat embryos 16, 18, 19, 20, and 21 days old were obtained; the embryological age was estimated after overnight mating.

Carcinogenesis

Fifty rats were given s.c. injections of 15 mg of DMH per kg body weight (Merck, Darmstadt, Federal Republic of Germany) freshly dissolved in 0.05% EDTA and adjusted to pH 6.5 with NaHCO₃ every week for 28 weeks (15). Seventeen rats received only buffer solution. Seven days after the last injection, one or two treated rats were killed each week from the start of the experiment by cardiac puncture under ether anesthesia; one control rat was killed every 2 weeks.

Histochemistry

The distal colon was excised, opened for macroscopic examination, pinned on cork, fixed for 2 hr in a box containing 95% ethanol, coiled up into “Swiss rolls” (9), fixed again according to the method of Sainte-Marie (19), and then embedded in paraffin.

Four serial sections were cut 3 μm thick with a R-Jung autocut (Heidelberg, Federal Republic of Germany). One section was stained with hematoxylin/eosin/safranin; a second was stained with high-iron diamine/Alcian blue to visualize both sialo- and sulfated mucins (22), and the final 2 sections were kept for immunohistological investigations.

Immunohistochemistry

Rabbit antiserum was prepared against M1 antigens isolated from a human ovarian mucinous cyst of the pure endocervical type as described previously (5). Before use, it was absorbed by 3 successive incubations for 1 hr at 20°C under agitation with glutaraldehyde-polymerized normal human plasma (1) until it gave no reaction with normal human serum in immunodiffusion. It was then adsorbed with a panel of human RBC (1 volume of RBC for 1 volume of antiserum at 20°C for 30 min and at 4°C for 12 hr) until no hemagglutination occurred against A, B, or O RBC (3). This anti-M1 serum then reacted with mucus of gastric surface epithelium using the immunoperoxidase method as described previously (4); it also quite clearly revealed components associated with rat surface epithelium of gastric mucosa, but in order to make it specific for gastric mucosa in rat, it was necessary to absorb it with aqueous extracts of rat cecum and colon. Scrapings of rat cecum and distal colon were made in water and then pounded with a vortex and lyophilized; 100 mg dry weight of each lyophilized extract were used to absorb 1 ml of anti-M1 serum. Thus, with the immunoperoxidase method, M1 antigens associated with goblet cells were shown to be present after 2 weeks of 1,2-dimethylhydrazine treatment in histologically normal mucosa and then in 78% of mucinous hyperplasia and polyoid-like glands, in 54% of hyposecreting glands, and in 58% of dysplasias, Grades 1 and 2, in two of 12 dysplasias, Grade 3, and in five of five transitional mucosas adjacent to carcinoma. The production of sialomucins associated with M1 antigens was often seen in the same histological lesion, although not always associated in the same goblet cells. The number of these histological lesions as well as the production of sialomucins associated with M1 antigens increased with the number of injections. Thus, these antigenic changes of an oncofetal nature can be regarded as early modifications of goblet cell differentiation in colonic mucosa subjected to chemical carcinogen.
method, anti-M1 serum stained rat surface gastric epithelium, whereas the remainder of the gastrointestinal tract and, particularly, the distal colon was unstained.

An indirect immunoperoxidase method was used. Anti-M1 serum absorbed as described was diluted 10-fold in PBS (0.9% NaCl in 0.01 M potassium phosphate buffer) and applied for 1 hr on the section; after 3 rinses with PBS, sheep antiserum against rabbit IgG (heavy and light) labeled with peroxidase (Institut Pasteur Production, Ville d'Avray, France) was applied at 1/100 dilution for 45 min; the section was washed 3 times with PBS, and peroxidase activity was revealed according to the method of Graham et al. (12) using aminothiol carbazol.

Before microscopic examination, cell nuclei were stained with hematoxylin for 1 min.

The specificity of staining was controlled by different absorptions; lyophilized human ovarian mucinous cyst of the pure endocervical type rich in M1 antigens or scrapings of rat gastric mucosa (250 mg each, dry weight, for 1 ml of anti-M1 serum) prepared as described, higher for colonic mucosa, completely suppressed rat gastric mucosa staining; on the contrary, absorption with lyophilized normal rat serum, liver, or scrapings of rat colon or cecum (500 mg each, dry weight, for 1 ml of anti-M1 serum) had no effect on gastric mucosa staining.

Histopathology

Morphological lesions were grouped into 5 categories, described as follows.

Epithelial Hyperplasia. The crypt of the gland is longer; the lumen is dilated; and the goblet cells are more numerous, taller, and distended with mucus.

"Polypoid-like" Glands. The absorptive cells are of various heights, giving the lumen a serrated appearance; in a human hyperplastic polyp, there is no cytonuclear modification; we have called such a lesion a "polypoid-like gland."

Glands with Hyposecretion and Dilated Lumen. The crypt may be longer, the lumen is dilated, and the goblet cells are very scarce or absent; the absorptive cells have a more eosinophilic ground-glass cytoplasm.

Dysplasia. As defined by 3 modifications (cellular atypia, abnormal differentiation, and disorganized mucosal architecture), we have graded the dysplasias 1, 2, or 3, according to the method of Nagayo (16).

With Grade 1 dysplasia, the nucleo/cytoplasmic ratio is increased, there is a decrease in mucus production, and the glands are dilated.

With Grade 2 dysplasia, the same modifications as in Grade 1 are seen as are stratification of the nuclei, which are sometimes found in the apical area of cells; a greater decrease in mucus production; and considerable dilation.

With Grade 3 dysplasia, the nuclei are irregular in shape and size, and some mitosis can be seen; mucus is scarce or absent, and glands have an irregular structure with back-to-back fusion.

Transitional Mucosa. Found adjacent to adenocarcinoma, it was histologically defined as often being thicker with elongated, branched crypts and dilated goblet cells with increased sialomucin secretion and decreased or absent sulfomucin (10).

RESULTS

Production of M1 Antigens by Colonic Mucosa during Carcinogenesis

The study was performed only on the distal part of the colon, where most human carcinomas arise; in addition, this distal part is histologically quite similar in both humans and rats.

In Control Rats

The mucosa of the distal colon produced only sulfated mucins and did not contain M1 antigens. None of the 17 control rats showed glands with a modification in differentiation or in their architecture.

In Treated Rats

M1 antigens were first detected in glands which seemed "normal," i.e., without detectable histological modifications, using hematoxylin-eosin staining (Fig. 1). A gland was considered positive when one of its cells was positive, but the number of positive cells could vary from one gland to another. The first positive gland was seen after one injection of DMH, and this number increased until the end of the experiment (34 weeks) (Table 1). Parallel to this, "normal" glands producing sialomucins were generally observed in lesser quantities. The production of M1 antigens and of sialomucins in the same gland was observed in 10 to 16% of these glands. In such cases, the 2 modifications could be seen in the same goblet cell or in different cells within the same gland.

M1 antigens were later observed in glands with histological modifications.

Epithelial Hyperplasia. The first gland showing epithelial hyperplasia was observed after the second injection. This number regularly increased from one to 6 per colonic section (mean value) until the 34th week (Table 2). Table 3 shows that 78% of glands with epithelial hyperplasia produced M1 antigens associated with sialomucins for 63% of them (Fig. 3, a and b); 11% showed no modification and produced only sulfated mucins.

Polypoid-like Glands. The first glands were seen after the 15th injection; their number remained low, as seen in Table 2, with very little increase with time. Fifteen of the 19 polypoid-like glands counted produced M1 antigens, and 13 of these 15 also produced sialomucins (Fig. 2).

Glands with Hyposecretion and Dilated Lumen. The first gland was observed after the fourth injection; the number increased from the tenth injection to reach 7 glands per colonic section (mean value) and then remained constant to the end of the experiment (Table 2). Fifty-four % of these glands produced M1 antigens with 42% also producing sialomucins (Fig. 4); 22% produced only sulfated mucins.

Dysplastic Glands. Dysplastic glands were first seen after the ninth injection; Table 2 shows that 5 dysplastic glands (mean value) could be observed in each colonic section from the 11th to 20th injections; this number then increased sharply, reaching 22 dysplastic glands per colonic section from the

<table>
<thead>
<tr>
<th>No. of M1 antigens positive</th>
<th>Sialomucins positive</th>
<th>M1 antigens and sialomucins positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk after the start of DMH treatment</td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>1-10</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>11-20</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>21-34</td>
<td>16</td>
<td>63</td>
</tr>
</tbody>
</table>
Antigenic Modifications in Colon of DMH-treated Rats

Table 2
Histological modifications during rat colonic carcinogenesis

Four different histological lesions as defined in "Materials and Methods" were counted throughout the experiment.

<table>
<thead>
<tr>
<th>Wk after the start of DMH treatment</th>
<th>No. of mucosa counted</th>
<th>No. of glands/colonic section</th>
<th>Mean of lesions counted/colon section</th>
<th>Range of lesions counted/colon section</th>
<th>No. of glands/colonic section</th>
<th>Mean of lesions counted/colon section</th>
<th>Range of lesions counted/colon section</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>20</td>
<td>1</td>
<td>0-5</td>
<td>0</td>
<td>1</td>
<td>0-2</td>
<td>0-6</td>
</tr>
<tr>
<td>11-20</td>
<td>14</td>
<td>3</td>
<td>0-6</td>
<td>&lt;1</td>
<td>7</td>
<td>3-12</td>
<td>0-4</td>
</tr>
<tr>
<td>21-34</td>
<td>16</td>
<td>6</td>
<td>0-15</td>
<td>1</td>
<td>1</td>
<td>0-16</td>
<td>0-10</td>
</tr>
</tbody>
</table>

Table 3

M1 antigens and sialomucins in histological lesions during rat colonic carcinogenesis

<table>
<thead>
<tr>
<th>Type of mucins produced with</th>
<th>No. of observed glands with</th>
<th>Dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 specificity</td>
<td>Sialomucins</td>
<td>Epithelial hyperplasia glands</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>101 (63)*</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>24 (15)</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>17 (11)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>18 (11)</td>
</tr>
<tr>
<td>Absence of mucin production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. of lesions</td>
<td>160</td>
<td>19</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.

Modifications at Peyer's Patches. A number of colonic sections showed Peyer’s patches of various sizes. In 17 colonic sections of control rats, numerous glands near the 10 Peyer’s patches observed showed architectural dysplastic- or hyperplastic-like modifications upon hematin/eosin staining; however, they did not produce M1 antigens or sialomucins. In 50 colonic sections of DMH-treated rats, the glands near the 35 Peyer’s patches observed showed the same architectural modifications but also showed changes in differentiation. They very often produced M1 antigens and sialomucins. These modified glands were not counted in the above results, as their number depended on the presence and the size of the Peyer’s patches of the section.

Production of M1 Antigens by Colonic Mucosa of Embryos and Young Rats

No goblet cells could be seen in colonic mucosa of 16- and 18-day-old embryos. At Days 19 to 20, goblet cells were observed, stained with periodic acid-Schiff and producing sulfated and sialylated mucins; at the same time, some goblet cells produced M1 antigens (Fig. 8). A number of different organs, present on the sections, were tested simultaneously: stomach; duodenum; small intestine; liver; kidney; pancreas; lungs; heart; thymus; spleen; endometrium; endocervix; and salivary glands. None of them produced M1 antigens at Days 19 to 20 before birth.

One day after birth, the colonic mucosa was identical to adult mucosa in mucin content; all goblet cells produced acidic sulfated mucins; sialomucins and M1 antigens were absent.

DISCUSSION

Using an immunohistological method, we were able to detect changes in the antigenic specificity of mucins produced by goblet cells of rat colonic mucosa during carcinogenesis; we observed the production of mucins called M1 antigens, with an antigenicity which is usually specific to gastric mucins.

M1 antigens appeared very early in the colonic mucosa of DMH-treated rats, before any architectural modification of the glands could be seen. Similarly, Chiu et al. (6) have shown change in the immunospecificity of the nuclear antigens in chromatin of rat colonic mucosa after only 4 weeks of treatment with DMH; they found also that antisera prepared from rat colon adenocarcinoma cross-react with human and mouse colon adenocarcinomas.

We noticed also that some glands produced sialomucins. In other tissues, changes in the antigenic specificity of mucins were also observed; for example, in the lungs, heart, thymus, spleen, endometrium, endocervix, and salivary glands.

Colonic Adenocarcinoma. The first adenocarcinoma was seen after 27 injections. Four invasive adenocarcinomas, from Stages B to D according to Dukes’ classification, one in situ adenocarcinoma, and one mucinous adenocarcinoma were studied. The in situ adenocarcinoma as well as one invasive adenocarcinoma did not show production of M1 antigens. The other 4 adenocarcinomas produced M1 antigens in 50 to 70% of their glands in either surface or the submucosa and muscle (Fig. 6).

Transitional Mucosa. Transitional mucosa was studied adjacent to 3 invasive adenocarcinomas, one in situ adenocarcinoma, and one signet-ring cell carcinoma. All of the 5 produced M1 antigens.

Fifty glands were counted: 26 of them produced M1 antigens in 20 to 100% of their goblet cells (Fig. 7); 49 of 50 glands produced sialomucins.
the same way, Shamsuddin and Trump (20) observed a predominance of sialomucins in morphologically normal crypts in rat colonic mucosa during histogenesis of azoxymethane-induced carcinoma. All this suggests an early cellular response of the colonic mucosa to the carcinogen. It is probable that these alterations either progress to cancer or return to normal.

The number of glands producing M1 antigens regularly increased with time, even after the end of DMH injections.

In our study, production of M1 antigens and production of sialomucins were not systematically associated in the same cell and in the same gland; we were dealing with 2 different modifications. Thus, the immunohistochemical method used provides new information on goblet-cell differentiation.

However, the production of M1 antigens associated with the production of sialomucins was more often found in a gland showing histological modification than in a gland which seemed histologically “normal.”

Seventy-eight % of glands with epithelial hyperplasia (the number of which increased regularly throughout the experiment) produced M1 antigens; 63% of them produced M1 antigens associated with sialomucins. These results are in contrast with the observations of Filipe (9) who saw no alterations in the mucin composition in the areas of hyperplasia; however, we often observed epithelial hyperplasia and dysplasia in the same focus of mucosa, which seems to indicate that the DMH-subjected mucosa can react by various changes in architecture and differentiation.

Sixty-eight % of polyoid-like glands produced M1 antigens and sialomucins. In humans, the hyperplastic polyps corresponding in rat to the polyoid-like glands were considered to be benign lesions; however, there are recent reports of coinciding hyperplastic and dysplastic changes, suggesting a relationship between these growth disorders (8, 24). Such observations are similar to our own in the rat.

Dysplastic glands appeared only at Week 9 and showed a sharp increase in number after Week 21, just before the tumors appeared. Forty-seven % of dysplasias of Grades 1 and 2 produced M1 antigens and sialomucins; this percentage remained constant throughout the experiment. Twenty % of the dysplastic glands produced sulfomucins only, and 9% had no mucin production. In Grade 3 dysplasia, the majority of the glands were nonproducing (75%); moreover, there was a decrease in M1 antigen production. Since we found that 4 of 6 rat colonic carcinomas produced M1 antigens, it seems that during carcinogenesis, neoplasia passes through an undifferentiated stage of very little secretion (Grade 3 dysplasia).

Several authors have noticed glandular modifications near Peyer’s patches; these observations were carried out on histological criteria. Ward (23) and Rogers et al. (18) thought that carcinoma arose rather frequently in or adjacent to lymphoid follicles and that the early changes observed in glands might be precursors of cancer. Pozharisski (17) did not agree, since he observed such modifications in control rats. Our work shows that, in treated rats only, histological changes are associated with immunological changes; this would seem to be in agreement with Rogers’ hypothesis, since we always found these changes when a Peyer’s patch was seen on the section; however, the number of modified glands does not seem to increase with the number of injections, and this study does not permit a definitive conclusion.

The number of histological lesions described increased with time, as did the total number of glands secreting M1 antigens. We thus feel that M1 antigens are associated with the early carcinogenic process.

What is the nature of such a modification? As already described by others (13, 14), we also detected the first colonic goblet cells producing sulfomucins and sialomucins at Day 19 or 20 of embryogenesis; we have demonstrated that they also produce M1 antigens. One day after birth, the goblet cells produced only sulfomucins, and the M1 antigens had disappeared, as later seen in adult rat colonic mucosa. Thus, M1 antigens which have gastric specificity in the adult are found to have colonic specificity in the fetus; the same observation was seen in humans (2).

In our experiment, 4 of 6 adenocarcinomas produced M1 antigens as did 5 of 5 transitional mucosas. In humans, 29% of colonic adenocarcinomas showed the presence of M1 antigens. We therefore feel that M1 antigens have an oncofetal behavior.

In conclusion, using an immunohistological method, it has been shown that M1 antigens associated with the mucous cells of gastric epithelium could be regarded as early oncofetal markers in the colonic mucosa during carcinogenesis.

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Fig. 1. Histologically normal mucosa after 6 weeks of DMH injections, showing a gland with 2 cells producing M1 antigens (arrows). Immunoperoxidase, × 250.

Fig. 2. A polypoid-like gland after 20 weeks of DMH injections with several cells producing M1 antigens (arrows). Immunoperoxidase, × 250.

Fig. 3. Two serial sections of mucosa after 21 weeks of DMH injections showing 2 glands with mucinous hyperplasia (top left). a, producing M1 antigens. Immunoperoxidase, × 100. b, producing both sialomucins stained gray (arrow) and sulfomucins stained black. High-iron diamine/Alcian blue, × 100.
Antigenic Modifications in Colon of DMH-treated Rats

Fig. 4. Two glands with hyposcretion and dilated lumen with cells producing M1 antigens (arrows) and intraluminal deposit after 13 weeks of DMH injections. Immunoperoxidase, × 250.

Fig. 5. A dysplastic gland, Grade 1, after 19 weeks of DMH injections with cells producing M1 antigens (arrows). Immunoperoxidase, × 250.

Fig. 6. Several glands and isolated cells in a well-differentiated adenocarcinoma producing M1 antigens (arrows). Immunoperoxidase, × 250.
Fig. 7. A typical branched gland in transitional mucosa adjacent to an adenocarcinoma with some cells producing M1 antigens (arrows). Immunoperoxidase, × 250.

Fig. 8. Colonic mucosa of a 21-day-old embryo producing M1 antigens in some cells and also in lumen (arrows). Top, immunoperoxidase staining. Bottom, negative control unstained with anti-M1 serum absorbed with extracts rich in M1 antigens.Lower half on a serial section. × 250.
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