Immunohistological Study of Precancerous Mucus Modification in Human Distal Colonic Polyps

J. Bara, O. Languille, M. C. Gendron, N. Daher, E. Martin, and P. Burtin

ABSTRACT

The M1 antigens associated with gastric fucomucins, oncofetal markers of the distal colonic mucosa, were demonstrated to be more closely associated with adenomas [92 of 139 (66%)] than with invasive adenocarcinomas [27 of 218 (12%)]. They were always expressed in tumors containing the M3 antigen normally associated with the intestinal mucus. The M1 antigens, present in 100% of hyperplastic polyps (30 of 30), were not specific for a particular histological type of adenoma but were found to be more closely associated with those showing a villous differentiation (41 of 47 (87%)) than with those having a tubular pattern (51 of 92 (55%)). The presence of these M1 antigens depended neither on the size nor on the degree of cytological atypia of the nodular adenomas. However, M1 antigens were found in 94% of the adenomas (35 of 37) concomitant with adenocarcinomas; in contrast, only 56% of adenomas (55 of 102) observed on noncancerous mucosa contained these M1 antigens. As already demonstrated during rat colonic carcinogenesis, mucus modification characterized by the presence of M1 antigens could represent early molecular changes occurring before malignant transformation related to a chemical carcinogen. These M1 antigens might be regarded as early precancerous markers of an oncofetal type, associated with human distal colonic mucosa.

INTRODUCTION

Mucin alteration has been described using histochemical methods in precancerous rat colonic mucosa during DMH\(^4\) carcinogenesis (15) and in humans in transitional mucosa (16). Such modifications can also be observed by electron microscopy in transitional mucosa (10) and adenomas (28). On the other hand, using immunohistological methods, other mucus modifications have been observed, such as the presence of A or B blood group substance (8, 22) or the accessibility of galactose to the PNA (7). Some of these alterations are regarded as oncofetal (8, 16). Recently, we characterized M1 antigens associated with fucomucins (3, 5, 6) and isolated from an ovarian mucinous cyst fluid of a pure endocervical type (3) according to the classification of Fenoglio et al. (14). The M1 antigens are present in fetal but not adult distal colonic mucosa (2) showing an oncofetal behavior, and they are also found in 29% of adenocarcinomas (5) and in 57% of their adjacent mucosa (2). More recently, we demonstrated the presence of such M1 antigens in the rat distal colonic mucosa 2 weeks after the first injection of DMH (11), suggesting that these M1 antigens are early markers of precancerous colonic mucosa. This led us to study human colonic polyps. Currently, in humans, several criteria such as size, degree of epithelial dysplasia, and pattern of growth (presence of villous differentiation) are useful for assessing the malignant potential of these polyps (25), but no precise markers exist. Thus, we have investigated herein whether the M1 antigens can be regarded as putative markers of the precancerous state in humans.

MATERIALS AND METHODS

Tissues

Normal Colonic Mucosa. These were obtained from kidney donors. Autopsies were performed on 16 patients (12 men and 4 women) within 1 to 3 min after cessation of their cardiopulmonary function. Most patients had been in their fourth decade of life (mean age, 35.1 years), their ages ranging from 17 to 54 years. All had been free of any known neoplastic disease prior to trauma. Tissue samples measuring about 10 x 1 cm were taken off the distal part of the colon.

Colonic Tumors. Two hundred eighteen adenocarcinomas and 37 associated polyps from distal colons surgically resected for cancer were obtained from the Clinique Chirurgicale de la Porte de Choisy, Paris, France. One hundred two polyps were obtained from distal colonic polypectomies of patients without detectable cancer (55 polyps from the Hôpital Antoine Béclère, Clamart, France, and 47 polyps were from the Centre Hospitalier Régional d’Amiens, Amiens, France). Samples of tumors were taken no more than 1 hr after fresh resection and immediately fixed in 95% ethanol. For the adenocarcinomas, 2 or 3 fragments measuring 1 sq cm were cut off the bulk, together with a sample of adjacent nonmalignant mucosa. In this study, we did not consider tumors from the proximal colon, i.e., the part of the colon located between the cecum and the left flexure, nor adenomas from patients with familial polyposis or Gardner’s syndrome.

Immunohistochemistry

Antigens. The antigens studied here are associated with the mucus of gastrointestinal epithelial cells. The first, called M1 antigens, are associated with gastric fucomucins (3). They were isolated from a mucinous ovarian cyst of a pure endocervical type according to the classification of Fenoglio et al. (14). We had already shown that these antigens are identical to the antigens of gastric surface epithelium (6). Another antigen, called the M3 antigen, is associated with sulfomucins or sialomucins of intestinal goblet cells (4, 5) and was isolated from normal colonic mucosa. Preparations of M1 and M3 antigens were obtained by chromatography on Sepharose CL 6B (Pharmacia, Uppsala, Sweden) (5).

Antisera. Anti-M1 and -M3 sera were obtained by immunization of rabbits with the preparations of M1 and M3 antigens, as already described (5). Such antisera were absorbed with normal human plasma and a panel of human RBC, and the absence of reactivity of these antisera against plasma antigens and blood group substances was controlled using, respectively, immunodiffusion and hemagglutination. Just before the IP test, the anti-M3 serum was absorbed with lyophilized crude extract of gastric mucosa (500 mg/ml). Thus, using the IP method,
muscularis mucosae [Stages B and C of Duke (12)] were examined for cinomas. In adenocarcinomas, only the invasive areas infiltrating the nocarcinomas; 5 signet-ring cell carcinomas; and 4 undifferentiated car were observed in our study: 193 adenocarcinomas; 16 mucinous ade- the WHO classification (26). Among the 7 types described, only 4 groups characterized by serrated borders and focal hypermature goblet cells observed. They were then classified into tubulovillous type I, if villous tubulovillous adenomas according to the percentage of villous areas WHO classification (26), slightly modified as follows. Because pure villous Classification of Tumors adenomas do not exist, it was preferable to consider 2 groups of nodular adenomas were observed and then subdivided according to the spreading according to growth aspect. Thus, 23 spreading and 116 polyps in a given category divided by the number of these polyps. mean of the IP score was calculated as the sum of the IP score of all tumors was evaluated on fresh resections.

IP Method

Normal colonic mucosa fragments measuring 10 x 1 cm were pinned on cork, fixed for 2 hr in a box containing 95% ethanol, coiled up into “Swiss roll” (15), and then, like the tumor samples, incubated overnight in the same fixator and embedded in paraffin according to the method of Sainte-Marie (36). Serial sections 2 µm thick were cut from the tissue blocks with an Autocut (R. Jung, Heidelberg, Federal Republic of Ger- many), dehydrated in successive batches with xylene and ethanol, and stained by the indirect IP technique. The first layer was either a control serum or the anti-M1 or -M3 serum at 1/50 dilution in 0.9% NaCl solution in 0.1 M potassium phosphate buffer and was incubated for 30 min. The second layer was a sheep antiserum against rabbit IgG (H + L) labeled with peroxidase (Institut Pasteur Production, Ville d’Avray, France) which was applied at a 1/100 dilution for 30 min. Peroxidase activity was revealed using aminoethylcarbazol (Sigma) according to the method of Graham et al. (19). Before microscopic examination, cell nuclei were stained with 1% hematein for 1 min. Inhibition of the immunological reaction was performed by incubation of diluted antisera with a solution containing the M1 or M3 antigens, isolated by chromatography on a Sepharose CL 6B. The antigen/antibody solution was incubated for 30 min at room temperature and centrifuged before the IP test.

A tumor was regarded as positive for a given M antigen if 10% of the tumoral areas was stained with this anti-M serum. When both the M1 and M3 antigens were present in the same tumor, the percentage of positive areas was estimated for each antigen. In this case, it was necessary to delineate the shape of each positive area and to estimate the positive surface of each anti-M serum. The M antigen occupying the larger surface was considered as predominant.

M1 staining was evaluated on a semiquantitative scoring system; a score of 0, 1, 2, or 3 referred, respectively, to 0 to 10%, 10 to 50%, 50 to 75%, or more than 75% of M1-positive adenomatous areas. The mean of the IP score was calculated as the sum of the IP score of all polyps in a given category divided by the number of these polyps.

Classification of Tumors

Adenomas. Adenomas were first divided into 2 groups: nodular and spreading according to growth aspect. Thus, 23 spreading and 116 nodular adenomas were observed and then subdivided according to the WHO classification (26), slightly modified as follows. Because pure villous adenomas do not exist, it was preferable to consider 2 groups of tubulovillous adenomas according to the percentage of villous areas observed. They were then classified into tubulovillous type I, if villous areas represented less than 50% of their surface, and tubulovillous type II, if the tumoral areas contained 50% or more villous differentiation.

Hyperplastic (Metaplastic) Polyps. These were arranged according to the WHO classification (26); they showed hyperplastic epithelium characterized by serrated borders and focal hypermature goblet cells (see Fig. 4, arrows).

Adenocarcinomas. Adenocarcinomas were classified according to the WHO classification (26). Among the 7 types described, only 4 groups were observed in our study: 193 adenocarcinomas; 16 mucinous adenocarcinomas; 5 signet-ring cell carcinomas; and 4 undifferentiated carcinomas. In adenocarcinomas, only the invasive areas infiltrating the muscularis mucosae [Stages B and C of Duke (12)] were examined for the presence of M antigens.

Grading of Dysplasia. Dysplasia was defined according to the 3 following criteria (27): (a) a change in the structure of cells, particularly of the nucleus; (b) a disturbance in differentiation, i.e., cellular function; and (c) a structural disorganization of the tissue. According to the description proposed by Martin et al. (24), dysplasias were classified into mild (Grade 1), moderate (Grade 2), and severe (Grade 3). Thus, ade- nomas could be ranked into Grades 1 or 2 if two-thirds of their glands showed Grades 1 or 2 dysplasia, respectively.

In this study, fociuses of severe dysplasia (Grade 3), carcinoma in situ, and microinvasive carcinoma without invasion across the line of the muscularis mucosae [Stage A of Duke (12)] observed in nodular adenomas were grouped together under the heading, “lesions including dysplasia 3-cancer sequence.”

Histochemistry

Paraffin sections were stained with hematoxylin/eosin/safran and Al- cian blue. They were used for histological classification and estimation of the mucosecretory tumoral areas.

Measure of Tumor Size

The diameter of nodular adenomas was measured using a centimeter on paraffin sections. For spreading surface adenomas, the perimeter of the tumors was evaluated on fresh resections.

Statistical Analyses

Tumor size is the only truly measurable variable in such investigations, whereas IP score and epithelial dysplasia grades are due to the operation of ranking. Statistical analyses must therefore be adjusted to the limita- tions given for the ordinal scale, as already used with other colon markers (34). Thus, the χ² test and the coefficient of correlation were applied for the nodular adenomas: correlation of size to IP score; and IP score to the degrees of epithelial dysplasia. The Student t test was used to compare both groups of adenomas (spreading or nodular), both histological types (tubular or tubulovillous), and adenomas from incidental finding or with concomitant adenocarcinomas.

RESULTS

M1 Antigens in Normal Distal Colonic Mucosa

Of the 16 different distal colonic mucosae studied here, 14 samples did not react with the anti-M1 serum. One colonic mucosa showed a patch of 5 glands forming a mucinous hyper-plasia and containing 50% of positive goblet cells. The mucosa around it was negative. The last one showed only one strongly positive goblet cell located deep within the Lieberkühn glands. Thus, the presence of M1 antigens was exceptional in normal distal colonic mucosa.

M1 Antigens in the Different Histological Types of Colonic Tumors

Adenomas

Table 1 shows the percentage of M1-positive tumors accord- ing to the histological type of adenoma; mean IP scores are reported for each group. Eighty-seven % of adenomas showing a villous pattern were positive (mean IP score, 1.7) in contrast to 55% of tubular adenomas (mean IP score, 0.93). Such a difference between the 2 groups is statistically significant (p < 0.001).

Seventy-two of 116 nodular adenomas were M1 positive, showing a mean IP score of 1.0. These M1 antigens were essentially located in the cytoplasm of tumoral goblet cells and in the lumen of the glands. These antigens were mainly associ- ated with mucinous inclusion of these mucosecreting cells (Fig. 1).

In 5% of M1-positive adenomas, goblet cells were negative,
but tumoral cells, showing features of both absorptive and secretory cells probably corresponding to intermediate or immature cells, produced these M1 antigens.

Positive glands tended to occur in focal clusters (Fig. 1, arrows) but were not homogeneously distributed throughout the polyp. The part of the adenomas showing histologically normal glands without detectable cytonuclear modification could sometimes be stained by the anti-M1 serum, showing strongly M1-positive goblet cells near M1-negative goblet cells (Fig. 2).

Spreading Adenomas. Twenty of 23 (86%) tumors were strongly stained and showed a mean IP score of 2.0. The M1 pattern was more homogenous than in nodular tumors, and focal clusters were infrequent. A typical pattern of these M1 antigens was observed in mixed glands containing both normal and neoplastic tissue (Fig. 3, arrows). The M1 antigens were associated primarily with the dysplastic glands (Fig. 3) more clearly showing abrupt changes observed between both normal and neoplastic tissue. Such a pattern was very rarely seen in the 116 nodular adenomas (3 cases). Only one spreading tumor showed M1 antigens predominantly in tumoral cells, which could be considered as intermediate or immature cells.

Hyperplastic Polyps

Thirty of 30 (100%) hyperplastic polyps were stained by the anti-M1 serum (Fig. 4) with a mean IP score of 2.8. Generally, 80 to 95% of goblet cells were strongly stained. No polyp containing less than 50% of positive goblet cells (IP score, 2) has been observed to date.

Adenocarcinomas

Table 2 shows M1-positive adenocarcinomas according to their histological type; 12% of the invasive adenocarcinomas were stained by the anti-M1 serum. The percentage of labeling of this group of malignant tumors was significantly different from the group of adenomas (p < 0.0005). The mean IP score of M1-positive adenocarcinomas was 1.6, and the mean for all adenocarcinomas was 0.18.

Association of M1 and M3 Antigens

Table 3 reports the association of these M antigens in adenocarcinomas. One hundred % of adenomas contained the intestinal M3 antigen with a mean IP score of 3. This M3 antigen showed a tissue localization pattern identical to that of the M1 antigens. Sixty-six % of them also contained the M1 as a minor antigen. In the invasive areas of adenocarcinomas, 47% of tumors produced the intestinal M3 antigen, and 12% contained both M1 and M3 antigens. Except in 2 tumors, the intestinal M3 antigen was found to predominate (Table 3).

M1 Antigens in Adenomas Containing Lesions Including the Dysplasia 3-Cancer Sequence

Three of 5 nodular adenomas containing such a lesion were tubular, and the 2 others showed a villous pattern. Their relatively small size comprised between 1.3 and 2 cm. One of them did not show dysplasia. Two adenomas showed dysplasia of Grade 1, and the 2 others, dysplasia of Grade 2. Four of 5 of these tumors contained M1 antigens (mean IP score, 1). The M1

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<td>Presence and association of M1 and M3 antigens in distal colonie tumors</td>
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antigens were not detectable in the lesion including the dysplasia 3-cancer sequence which produced the M3 mucus-associated antigen in a small amount.

**M1 Antigens in Adenomas Concomitant with Adenocarcinomas**

Table 4 shows that 56% of polyps growing on noncancerous colonic mucosa were M1-positive mucosa having a mean IP score of 1.57. In contrast, 94% of polyps found concomitant with colonic adenocarcinomas were M1 positive (mean IP score, 2.02). The difference between these 2 percentages was statistically significant ($p < 0.001$).

**M1 Antigens and the Size or Degree of Dysplasia of Adenomas**

Chart 1 shows the IP score for M1 of adenomas in relation to their size, and Chart 2 reports the degree of dysplasia in relation to the IP score for M1 antigens. For each tumor, the histological patterns were noted. For the nodular adenomas, we calculated the coefficient of correlation ($r$) between the size and the IP score ($r = 0.24$) and between the IP score and the degree of epithelial dysplasia ($r = 0.02$).

**DISCUSSION**

The presence of M1 antigens in the human distal colon characterizes mucus modifications of an oncofetal type. Such alterations of mucus are more closely associated with benign (66% of adenomas) than with cancerous lesions (12%) ($p < 0.0001$). This is a recent observation, since until now, the antigenic oncofetal behavior associated with mucus was thought to be associated with cancer (23). These mucus modifications are more comparable to those described using PNA (7) than to those characterized by the A and B blood group antigens (8), which are not present in the hyperplastic polyps and are not particularly associated with adenomas showing a villous pattern.

We had already demonstrated that the M antigens, which predominate in gastrointestinal adenocarcinomas, were the predominant antigens of normal tissue from which tumors arise (1, 4, 5, 32). Such observations remain true for adenomas in which the M3 antigen predominates.

In our most recent study (5) concerning the presence of M1 antigens in colonic adenocarcinomas using the immunofluorescence technique, we found 29% of the adenocarcinomas to be M1-positive tumors. In contrast, in the present study, we observed only 12% to be M1 positive. Such a difference can be explained by the fact that: (a) this study was restricted to invasive areas of adenocarcinomas (the remaining adenomas sometimes observed adjacent to adenocarcinoma and often M1 positive were not included in our results); and (b) adenocarcinomas of...
the proximal part of the colon, 50% of which are M1 positive, were likewise not counted in this study.

These M1 antigens appear to be putative markers for precancerous distal colonic mucosa for the following reasons. (a) They are more often associated with adenomas (86%), known to be possible precancerous lesions, than with adenocarcinomas (12%). (b) These antigens are more frequently present in the adenomas which show villous patterns (87%) than in those showing only tubular differentiation. Morson (25) claimed that the malignant potential of adenomas depended on their villous pattern. (c) Ninety-four % of the polyps, growing on mucosas containing adenocarcinomas, are M1 positive; in contrast, only 56% of those arising from noncancerous mucosas are positive. (d) During rat carcinogenesis, Decaens et al. (11) demonstrated that these M1 antigens appear in the colonic mucosa 2 weeks after the first injection of DMH, suggesting that these antigens behave as early precancerous markers.

In contrast to the above evidence in favor of precancerous markers, 3 arguments exist against such a conclusion. (a) The hyperplastic polyp, which is not regarded as a precancerous lesion but which is ranked as a tumor-like lesion according to the WHO classification (26), always contains these M1 antigens. (b) The malignant potential of adenomas depends on their size (25), and in our study, no correlation could be demonstrated between the size of tumors and their positivity for the M1 \( r = 0.24 \). (c) The malignant potential of these adenomas depends on their degree of atypia (25), and our results show that no correlation can be established between the increase in the degree of dysplasia and that of the IP score for M1 antigens \( r = 0.02 \).

These 3 points are discussed in detail below.

(a) Until now, the most important differential features between the hyperplastic and adenomatous polyps were expressed in terms of maturation, i.e., quantitative modifications. The former shows a hypermaturation pattern, and the latter, immature features (21). Using an immunohistological approach, we demonstrated a differentiation change, i.e., a qualitative modification characterized by the expression of fetal fucomucin-associated antigens. Such a differentiation is common to both of these histological types of polyp. Another mucus modification has already been observed in both of these lesions using PNA (7). On the other hand, during rat carcinogenesis, some authors observed glands with serrated epithelium showing the same histological pattern as that observed in human hyperplastic polyps (33, 37). For this reason, the hyperplastic polyp could be regarded as a putative marker for precancerous colonic mucosa without these lesions necessarily becoming cancerous. However, even this latter remark, i.e., the hyperplastic polyp considered as a nonneoplastic epithelial proliferation which does not predispose the patient to colon cancer, is questionable for the following reasons. Hyperplastic polyps are prone to occur more frequently than adenomatous polyps at anatomical sites where the highest frequency of cancer is observed, suggesting that these lesions are somewhat more sensitive markers for high risk than are adenomas (20). Next, there have been recent reports of coincidental hyperplastic and dysplastic changes (9, 13, 18). Finally, histological evidence of adenocarcinomas occurring in hyperplastic polyps has been demonstrated (9, 17). These 3 observations tend to prove that hyperplastic polyps can sometimes degenerate into cancer, although such a finding is the exception rather than the rule (9).

(b) It is generally admitted that the malignant potential, which is usually the adenoma-cancer sequence, is a slowly evolving process and that the risk of developing cancer in adenomas increases with the increasing size of adenomas (25). Nevertheless, some exceptions do exist, since in some small hemispherical adenomas (29, 31), the adenoma-cancer sequence could evolve rapidly. This second apparent contradiction cannot be used to refute that the M1 antigens are markers for precancerous lesions.

(c) More surprising is the absence of a correlation between M1 and the degree of dysplasia; this is at variance with results observed in using other antigenic markers (35) and with the conventional view that dysplastic modifications represent the primary changes leading to cancer. During rat carcinogenesis, M1 antigens appear just 2 weeks after the first DMH injection (11) and tend to disappear in the dysplasia (a delayed lesion), since only 58% of dysplastic glands are M1 positive. In humans, nearly the same percentage (63%) is found. Such comparable observations suggest that M1 antigens may be regarded as early markers of a precancerous state, which tend to disappear when dysplasias appear. This suggestion agrees with the mean IP scores, showing that the M1 antigens were more poorly expressed in lesions having a higher malignant potential or in malignant lesions, such as adenomas, containing lesions including the dysplasia 3-cancer sequence or adenocarcinomas (mean IP scores of 1 or 0.18, respectively) than in hyperplastic polyps or adenomas (mean IP scores of 2.8 and 1.6 to 2.1, respectively).

Our final conclusion is that mucous modifications of the distal colonic mucosa characterized by the presence of M1 antigens could be regarded as an early precancerous change of an oncofetal type. It is generally accepted (25, 30) that adenomas are important precursors of colorectal cancer, yet only a small percentage with become invasive carcinoma.

Our working hypothesis is now to determine whether the M1 antigens could be useful as screening tools for the estimation of the malignant potential of adenomas.

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