Intestinal MUC2 and gastric M1/MUC5AC in preneoplastic lesions induced by 1,2-dimethylhydrazine in rat: A sequential analysis

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Abstract. Our study was performed to sequentially analyze the expression of the intestinal mucin MUC2 and of the gastric mucin MUC5AC as indicators during progression of pre-neoplastic biomarkers in rat colon. F344 rats were sacrificed 2, 4, 8, 12, 24 and 36 weeks after injection of 1,2-dimethyl-hydrazine (DMH, 200 mg/kg, IP). The expression of MUC2 and of MUC5AC was studied by immunohistochemistry in preneoplastic lesions classified in two categories: histologically altered foci (HAF) and ß-catenin accumulated crypts (BCAC). HAF appeared 4 weeks after DMH injection. Their crypt multiplicity stagnated with time (3-4 crypts/foci) but gastric MUC5AC mucin was always observed in some goblet cells of the lesions of this category. In contrast, MUC2-immunostaining was not modified compared to the adjacent crypts. Double-immunofluorescence revealed that goblet cells which produced MUC5AC continued to express MUC2. In BCAC, crypt multiplicity and mucin expression strongly evolved with time. These lesions were observed only 8 weeks after DMH-injection. At this stage, 20% of BCAC showed a decreased MUC2 expression and 33% were MUC5AC immunopositive. At the 36-week point, 43% of BCAC had a reduced MUC2 staining and 90% were positive for MUC5AC. This immuno-positivity was often observed in all the cells of these lesions. Seldom, some BCAC were depleted at the same time in MUC2 and in MUC5AC. Similar alterations in mucin expression were observed in human colonic pre-neoplastic lesions. These findings suggest that a decrease in MUC2 expression and staining of MUC5AC in non-goblet-like cells predicts histo-logical progression of preneoplastic lesions.

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Introduction

Colon carcinogenesis evolves through epithelial cell de-regulation, and aberrant crypt foci (ACF) are thought to be the earliest identifiable preneoplastic biomarkers in human and in rodent models of carcinogenesis (1,2). ACF were originally identified and defined by their microscopic appearance in unembedded mucosa (3). Numerous studies have established ACF as a biomarker of cancer risk in carcinogen-treated rodents not only for screening effective chemopreventive agents but also for risk assessment of chemical components (4,5). The progression of ACF to adenoma and, subsequently to adenocarcinoma, parallels the accumulation of several biochemical alterations and mutations whereby a small fraction of ACF evolves to colon cancer (6). Recently, other colonic biomarkers, the ß-catenin accumulated crypts (BCAC), have been described in *en face* preparations of colonic mucosa in rodent models of carcinogenesis and have been suggested to be premalignant rather than preneoplastic (7,8). Cytoplasmic or nuclear expression of ß-catenin has also been observed in crypt foci of patients with colon cancer (9) and both ACF and BCAC are widely used as biomarkers for short-term colon carcinogenesis bioassays (10-12). Recently, mucin-depleted foci (MDF) have also been proposed as additional preneoplastic lesions in the process of colon carcinogenesis of azoxymethane-treated rats (13,14). MDF are characterized by absent or scarce mucus production, and are revealed by high-iron diamine alcian blue histochemical technique that highlights mucin glycoconjugates. Indeed, epithelial surfaces are coated by a viscoelastic mucus gel that lubricates and protects the mucosa from mechanical insults, colonization by pathogenic bacteria and their toxins, luminal proteases arising from bacterial and mucosal cells, and potential carcinogens. The viscoelastic properties of mucus are mainly determined by the presence of mucins, which are high molecular weight proteins extensively O-glycosylated. Epithelial mucins can be divided into two main classes: secreted, and cell surface-associated mucins. Secreted gel-forming mucins include MUC2, MUC5AC, MUC5B and MUC6, which are encoded by a cluster of genes on chromosome 11p15.5 in humans and on chromosome 7 in murine rodents (15,16). It has been demonstrated that each of

these mucins has a characteristic organ- and cell type-specific distribution (17). Normal stomach mucosa is thus characterised by the production of MUC5AC, primarily by the surface epithelial mucus cells, and of MUC6 by the gastric glands. MUC2 is the secreted mucin that is predominantly present in small and large intestine and is confined to goblet cells.

Contrary to this tissue-specific expression, altered mucin gene expression is a common feature of precancerous lesions and of cancer. In particular, a decrease of MUC2 expression has been found in poorly differentiated human colorectal tumor compared with normal mucosa (18-20) and these alterations in MUC2 have been associated with tumor progression in the colon. In support of this idea, Velcich *et al* (21) have also demonstrated that inactivation of the major intestinal mucin gene MUC2 in mice causes intestinal tumor formation with spontaneous progression to invasive carcinoma. In colonic carcinogenesis, alterations in secreted gel-forming mucins also include *de novo* expression of the gastric mucin M1/MUC5AC that is described as an early marker of human (22) and rat (23) carcinogenesis. This gastric mucin is expressed in human colorectal adenocarcinomas (20,24) and in rat colon during 1,2-dimethylhydrazine (DMH) or methyl-N'-nitroso-guanidine (MNNG)-induced carcinogenesis (25,26). Changes of the expression of MUC5AC and MUC2 are thus a hallmark of colorectal carcinogenesis and it is critical to explore at which steps of the carcinogenesis these alterations of MUC2 and MUC5AC are detectable, and how they are linked.

To evaluate the early changes in mucin expression related to colon carcinogenesis and to explore the relationship between secreted mucins and colorectal carcinoma, we followed by immunohistochemistry the evolution of expression of MUC2 and of M1/MUC5AC in histological colonic lesions of DMH-treated rats from 2 to 36 weeks after exposure to the carcinogen. This animal model provides a good opportunity to study the changes in the expression of secreted mucins during the sequential development of histological lesions. Further, this is a useful experimental model in that it mimics the human adenoma-carcinoma sequence.

Materials and methods

Animals and carcinogen treatment. Male Fischer-344 rats (3 weeks of age) were purchased from Harlan (Harlan France, Gannat) and acclimatized to laboratory conditions for 2 weeks before receiving DMH dihydrochloride (200 mg/kg body weight; Sigma Chemicals, St. Louis, MO, USA). The rats were randomized by weight to an experimental group and a control group (36 rats in each group), housed in plastic cages (6 rats/cage) with free access to tap water, and received a diet enriched in protein and lipid (2019 Teklad Global diet, Harlan France), *ad libitum*. This diet contained 19.20% crude protein (recommended AIN-93M: 12.58%) and 9.5% total fat (recommended AIN-93M: 4.0%). Rats were maintained at an environmental temperature of 20-24˚C and a 12-h light-dark cycle. Body weights and food intake were measured weekly. After acclimatization, animals in the experimental group were initiated with an intraperitoneal injection of DMH dissolved extemporaneously in PBS containing 1 mM EDTA and adjusted to pH 6.5 with sodium hydroxide. In the control group, rats were treated with intraperitoneal injection of vehicle. Rats treated with DMH (or not) were sacrificed 2, 4, 8, 12, 24 and 36 weeks after the injection of carcinogen in order to study the sequential appearance of histological lesions and the modification of mucin expression.

Evaluation of ACF in rat colonic whole-mount preparations. ACF were identified as described by the method of Bird (3,27). The colon was flushed with cold phosphate-buffered saline (PBS), opened along the longitudinal median axis, and fixed flat between 2 pieces of filter paper in 90% ethanol for 24 h at -20˚C. We only studied the transverse and distal colons which are localized just after the herring-bone pattern (proximal colon). The colonic tissues were stained with 0.2% methylene blue in PBS for 1 min, placed on a microscopic slide with the mucosal side up and observed through a light microscope at magnification x40. ACF were identified by their increased size, irregular and dilated luminal opening, and thicker epithelial lining and pericryptal zone. The number of ACF per colon and the number of aberrant crypts observed in each focus were recorded. Average crypt multiplicity was determined as the mean number of crypts/focus/colon. ACF in each group were further categorized into small (1-3 crypts/focus), medium (4-6 crypts/focus), large (7-9 crypts/focus) and extra-large (>9 crypts/focus) foci. One DMH-treated group (six rats) and one control group (six rats) were sacrificed at each time-point. All colons were scored by a single observer. After ACF evaluation, each colon was cut into four sections of equal lengths and embedded in *en face* paraffin preparation for histological analysis and for immunological characterization of mucin expression in pre-neoplastic lesions.

Human colonic tissue. Human colon tissue samples were taken not later than 1 h after surgery from patients with colonic adenocarcinoma. Strips of macroscopically normal colon adjacent to the tumor were cut from the entire length of each specimen as already described (25,28). Human colonic mucosae (strips of 1x10 cm) were dissected from the underlying muscularis mucosa, fixed flat between coded filter paper, immersed for 2 or 3 days in 90% ethanol as fixative, and then embedded in paraffin. The tissues were then examined for histological preneoplastic lesions.

Immunohistology

Antibodies. Immunohistochemical staining was carried out with a rabbit polyclonal anti-MUC2 antibody (H300, 1:250 dilution, Santa Cruz Biotechnology, CA, USA), a mouse monoclonal anti-rat M1/MUC5AC antibody [Mab 660, 1:50 dilution (25,29)], a mixture of eight mouse monoclonal anti-human M1/MUC5AC antibodies [PM8, 1:250 dilution (30,31)], a rabbit polyclonal anti-ß-catenin antibody (H-102, 1:100 dilution, Santa Cruz Biotechnology). The rabbit polyclonal anti-MUC2 antibody was directed against amino-acids 4880-5179 mapping at the C-terminus of Mucin 2 of human origin and was used for detection of Mucin 2 of human or rat tissue.

Immunohistochemistry. The *en face* preparations of the colonic mucosa were examined by using 4-μm-thick serial sections. The deparaffinized sections were rehydrated through graded alcohols at room temperature. Sections were stained

with hematoxylin for histological examination. PBS (pH 7.6) was used to prepare solutions and for washes between various steps. Immunohistochemical incubations were performed in a humidified chamber. Endogenous peroxidase activity was quenched with 20-min incubation in 5% H_2O_2/PBS. After washing slides, antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6.0) by microwaving. For rat or human M1/MUC5AC mucin immunohistochemical staining, microwave treatment was omitted. Slides were then washed three times in PBS, treated for 30 min at room temperature with 2% BSA and 10% horse-serum (blocking solution) and incubated for 30 min at room temperature with primary antibody against rat or human M1/MUC5AC, MUC2, or ß-catenin diluted in blocking solution. Sections were then washed with PBS and incubated with corresponding biotinylated secondary antibody (1:100, Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After washing, the sections were incubated with avidin-biotin peroxidase complex provided by Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. Color was developed with 3,3-diaminobenzidine (DAB) solution or Vector® SG substrate (all from Vector Laboratories). Sections were then counterstained with hematoxylin, cleared, and mounted. For each case, negative controls with primary antibody omitted were performed on serial sections. The positive control slides were normal colonic mucosa (MUC2) or normal gastric mucosa (M1/MUC5AC).

Immunofluorescence. In order to determine whether MUC2 and gastric mucin M1/MUC5AC were colocalized within mucin-producing cells in histological lesions, we used double-labeling fluorescence immunohistochemistry. The paraffin-embedded sections were deparaffinized in methylcyclohexane, and rehydrated through graded alcohols at room temperature. Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6.0) by microwave treatment. The sections were treated for 30 min in blocking solution (with 2% BSA and 10% fetal bovine serum) and then incubated for 30 min at room temperature with primary antibodies (rabbit anti-MUC2, H-300, 1:250; and mouse anti-M1/MUC5AC mucin, MAb 660, 1:50). The slides were then rinsed five times with PBS and immune complexes were revealed with anti-rabbit-FITC and anti-mouse rhodamine-conjugated antibodies (Jackson ImmunoResearch) at a dilution of 1:100. The slides were then rinsed, cleared and mounted.

Mucin histochemistry. To provide an estimate of depletion of mucus stores from goblet cells in some histological lesions and to visualize mucin depleted foci (MDF), alcian blue (pH 2.5) staining was performed, as described previously (32). Pieces of colon (2-2.5 cm) were sectioned at 4 μm, stained with alcian blue and then counterstained with hematoxylin. MDF were characterized by the absence or very limited production of mucins.

Statistical analysis. The overall evolution of the number of lesions was assessed using Kruskal-Wallis non-parametric ANOVA, and single comparisons at different times after DMH injection were assessed with the U test of Mann-Whitney. Differences with $p<0.05$ were considered significant. Data were analyzed with the Statview 4.57 software for Windows (Abacus concept, Berkeley, CA, USA) and are presented as mean ± SEM.

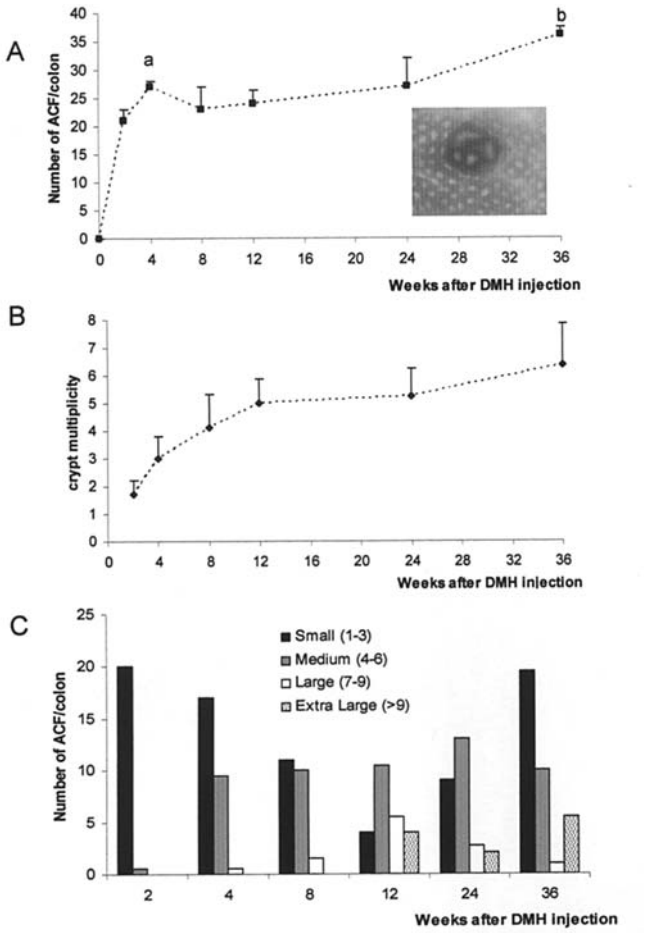

Figure 1. ACF in whole mount colon from 2 to 36 weeks after the injection of DMH. (A) Time course of the average numbers (mean ± SEM) of ACF per colon in rats treated with DMH. Male F344 rats were given a single injection of DMH (200 mg/kg body weight). Animals were sacrificed 2, 4, 8, 12, 24 or 36 weeks after the carcinogen injection and their transverse and distal colons were fixed in alcohol and stained with methylene blue. ACF were distinguished from normal crypts by their larger size and elliptical shape (inset, methylene blue-stained ACF consisting of 5 large crypts in the 12 weeks end-point assay). a, $p<0.05$ vs 2- or 8-week point. b, $p<0.05$ vs 8-week point. (B) Mean crypt multiplicity (± SEM) at every time-point. (C) Average numbers of ACF categorized as small, medium, large and extra large foci in the colons of rats treated with DMH.

Results

General observations in whole mount colon stained with methylene blue

Evolution of the number of ACF/colon. The temporal evolution of the number of ACF per colon is presented in Fig. 1A. ACF were detectable in carcinogen-treated animals at the first time-point of the study (2 weeks after DMH injection). ACF were stereoscopically distinguished from normal crypts by their darker staining and larger size, elliptical shape, thicker epithelial lining, and larger pericryptal zone (inset, Fig. 1A). A peak in the number of ACF per colon was observed 4 weeks after treatment with DMH (27±1 foci/colon, $p<0.05$ as compared to 2 or 8 weeks). Then, the number of ACF/colon

increased progressively to reach 36±2 foci per colon ($p<0.05$ as compared to 4 weeks) at the 36-week point. For every studied time, all DMH-treated rats had ACF, and no ACF was found in saline-treated rats throughout the experiments.

Evolution of the size of ACF. As shown in Fig. 1B, the crypt multiplicity steadily increased with time until the 12-week point. Then, the mean number of crypts per focus was quite stable. As observed in Fig. 1C, a biphasic evolution of the size of ACF was observed with time. At the first time-point, the majority of ACF were small (95.25% or 20 small ACF/colon) but this category of foci decreased to 16.70% (or 4 small ACF per colon) at the 12-week point. Then, the number of small foci increased again to reach 55.60% (or 20 small ACF per colon) to the 36-week time-point. Extra-large ACF were observed for the first time 12 weeks after treatment with DMH (16%). It is thus clear that the time affects the proportion of ACF with different crypt multiplicity. This progression of ACF morphology observed from the 2nd to the 36th week may correspond to the promotion step of colon carcinogenesis.

Analysis of histological preneoplastic lesions: histologically altered foci (HAF) and ß-catenin accumulated crypt (BCAC). Tissue sections in *en face* preparation were used for histological evaluation. Groups of crypts with morphological abnormalities were called histologically altered crypts (HAF) and were characterized either by their increased size, large crypts and wider lumens as compared with the surrounding crypts or by features of colonic dysplasia (increase of nuclear/cytoplasmic ratio, nuclear stratification, loss of nuclear polarity, structural abnormality of the crypts). We also searched groups of crypts which showed an abnormality at the level of their expression of ß-catenin by immunohistochemistry. The lesions showing accumulation of ß-catenin at the plasmatic or nuclear level were called ß-catenin-accumulated crypts (BCAC) (7,8). The few groups of crypts showing both types of abnormality (morphological and accumulation of ß-catenin) were classified in the BCAC. The average number of histological lesions per rat (HAF + BCAC) is shown in Fig. 2A. Histological lesions were detected only in the colon of rats treated with the carcinogen and became apparent 4 weeks after DMH treatment. The number of lesions per colon at this time-point was 2.8±1.5 (n=6). These lesions were mainly localized in the transverse colon. The number of lesions per colon then increased quickly to 11.8±3.1 at the 8-week point, the majority of the lesions being in the distal colon. The curve of the number of histological lesions per colon was then parallel to that obtained after counting the ACF in the whole mount colon. At the 36-week point, many small lesions were again observed in the transverse colon. Histological lesions of small size were the most frequent ones, whatever the time of the study and the colonic segment considered (Fig. 2B). In contrast, extra-large lesions, consisting of more than 9 crypts were seen only 8 weeks after DMH injection (Fig. 2B) and were mainly localized in the distal colon. The evolution of the relative amount of HAF and BCAC is summarized in Fig. 2C.

Evolution of the expression of the colonic mucin MUC2 and of the gastric mucin MUC5AC in HAF. The expression of

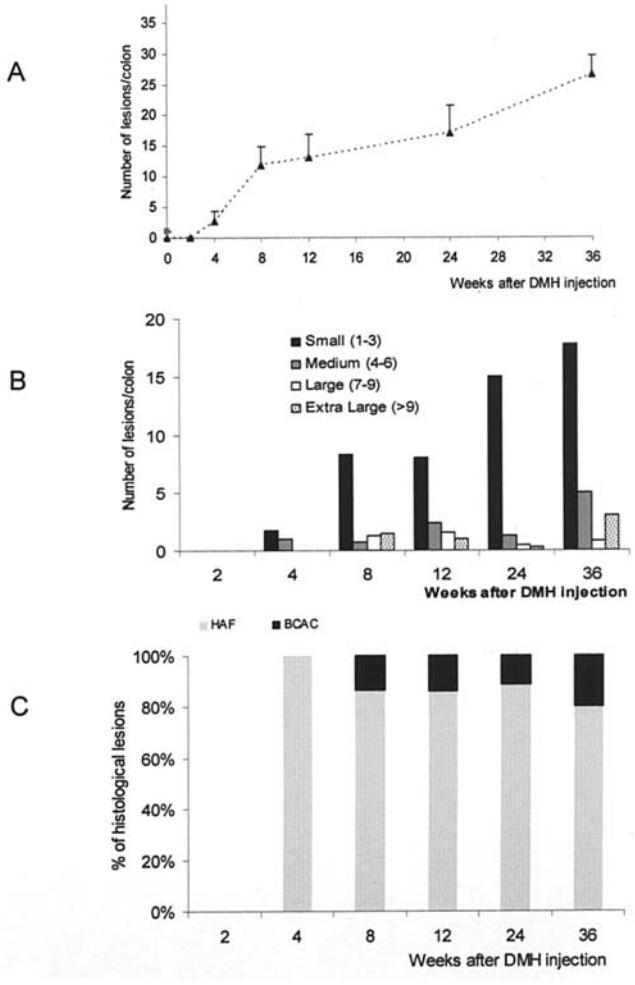

Figure 2. Analysis of preneoplastic histological lesions from 2 to 36 weeks after DMH injection. (A) Average numbers (mean ± SEM, n=6) of histological lesions (HAF + BCAC) per colon in *en face* preparation in rats treated with DMH. (B) Average numbers of histological lesions categorized as small, medium, large and extra large foci based on multiplicity in the colons of rats treated with DMH. (C) Incidence of BCAC and of HAF expressed as percent of histological lesions enumerated in the colon of DMH-treated rats.

M1/MUC5AC and of MUC2 was examined by immunohistochemistry in histologically altered crypts, and in the surrounding normal epithelium from 2 to 36 weeks after DMH treatment. As shown in Fig. 3A, the crypt multiplicity changed little with time. Fig. 3B shows the percentage of HAF with M1/MUC5AC staining at different time-points. It is to note that we observed gastric M1/MUC5AC immunoreactivity in numerous goblet cells of normal crypts two weeks after DMH treatment, i.e. before appearance of the histological lesions (data not shown). At the 4-week point, M1/MUC5AC immunoreactivity was only localized in HAF and most of these HAF strongly produced M1/MUC5AC in some goblet cells (Fig. 3C1). Whatever the time-point of the study, this production of gastric mucin deviated from the surrounding normal-appearing tissue and was not related to the presence of dysplasia or to the crypt multiplicity.

To determine whether the expression of the intestinal secreted mucin MUC2 was modified in preneoplastic lesions, immunohistochemical analysis was performed with a rabbit polyclonal anti-MUC2 antibody (H-300). Epithelial cells in

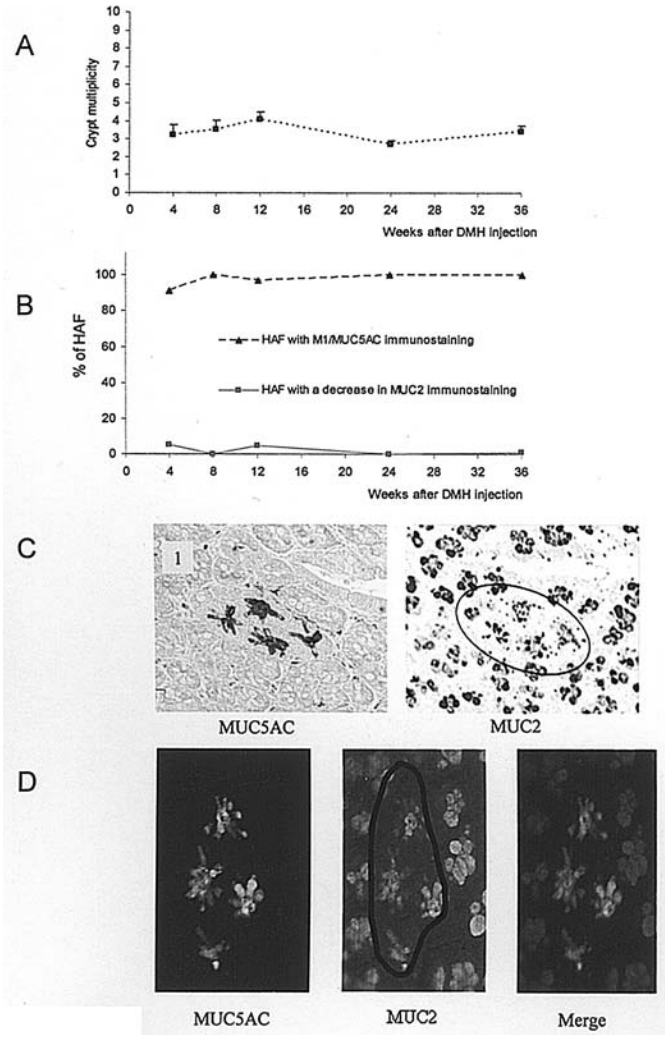

Figure 3. Analysis of MUC5AC and MUC2 expression in HAF from 2 to 36 weeks after DMH injection. (A) Time course of the mean crypt multiplicity (± SEM) of HAF. (B) Gastric mucin M1/MUC5AC and MUC2 expression as determined by immunohistochemistry in HAF from 4 to 36 weeks after DMH-injection. Results are expressed as percent of the total number of HAF. (C) Immunoperoxidase staining of M1/MUC5AC and MUC2 in HAF. (C1) Gastric M1/MUC5AC immunopositivity was detected in some goblet cells. Note that crypts in HAF were larger than adjacent normal crypts and that the immunopositivity was in goblet-like cells. (C2) Intestinal MUC2 immunopositivity. (D) Double-immunofluorescence staining for M1/MUC5AC and MUC2 was performed as described in Materials and methods.

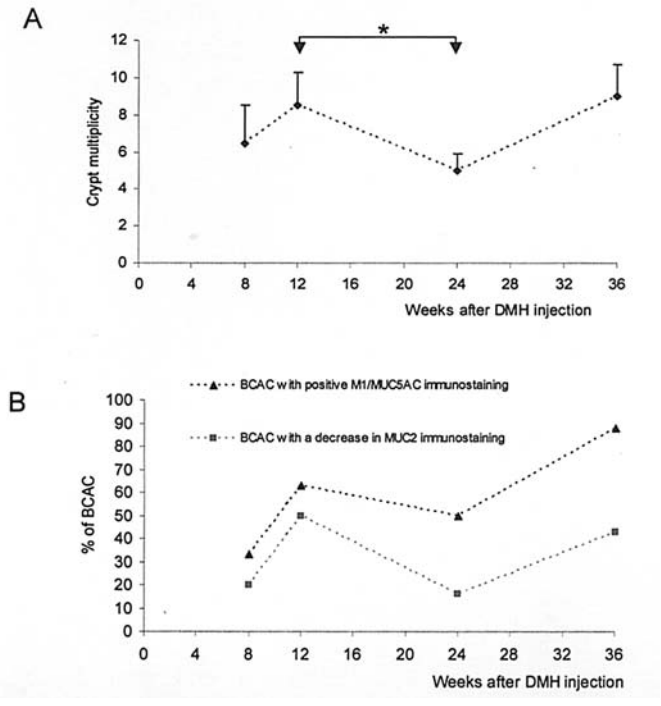

Figure 4. Analysis of M1/MUC5AC and of MUC2 expression in BCAC from 8 to 36 weeks after DMH injection. (A) Mean crypt multiplicity (± SEM) at every time-point. *p<0.05 vs 12-week point. (B) M1/MUC5AC and MUC2 expression as determined by immunohistochemistry in BCAC from 8 to 36 weeks after DMH-injection. Results are expressed as percent of the total number of BCAC.

normal colonic mucosa adjacent to HAF showed a strong cytoplasmic expression of MUC2 in goblet cells; this served as an internal positive control. MUC2 displayed a similar expression in most of the HAF than in the surrounding mucosa, despite the presence of dysplasia, the crypt multiplicity or the time-point of the study (Fig. 3C2). The expression of MUC2 was decreased in only very few HAF. Interestingly, by double-labeling immunofluorescence histochemistry for M1/MUC5AC and MUC2, we also showed that the goblet cells which produced M1/MUC5AC continued to express MUC2 (Fig. 3D). Histochemistry with alcian blue did not show differences between the HAF and the surrounding healthy tissue (data not shown).

Evolution of the expression of the colonic mucin MUC2 and of the gastric mucin MUC5AC in BCAC. As shown in Fig. 2C, BCAC were not observed before the 8-week point. At this time-point, BCAC represented 13.5% of histological lesions. At the 36-week point, 20% of histological lesions were BCAC. As shown in Fig. 4A, the crypt multiplicity of BCAC evolved with time with a decrease 24 weeks after DMH-injection. When data from all time-points were pooled, we noted that BCAC were predominantly (85%) detected in distal colon. We observed some BCAC with ACF-like appearance (enlarged crypt when compared with adjacent normal crypt). The others were with smaller crypts as compared with adjacent normal tissue.

Fig. 4B shows the pattern of gastric M1/MUC5AC and of colonic MUC2 in the BCAC. The proportion of BCAC which expressed M1/MUC5AC or which had a reduction in the expression of MUC2 varied according to the time-point of the study (Fig. 4B). At the 8-week point, 33% of the BCAC showed an immunopositivity for the gastric mucin and 20% had a decreased staining for MUC2. The majority of the first BCAC thus had an expression of secreted mucins which was similar to that observed in the healthy crypts. In contrast, M1/MUC5AC was expressed in 88% of the BCAC detected 36 weeks after DMH-treatment. The immunostaining for this gastric mucin was then observed mainly in cells which did not have the appearance of goblet cells (Fig. 5B and C). At this time-point, a sharp reduction of MUC2 immunopositivity was also observed in 43% of the BCAC as compared with the adjacent normal mucosa (Fig. 5A and B). Some BCAC (27% at 36-week point) were depleted at the same time in MUC2 and in M1/MUC5AC (Fig. 5A), and should probably be equivalent to the mucin-depleted foci (MDF)

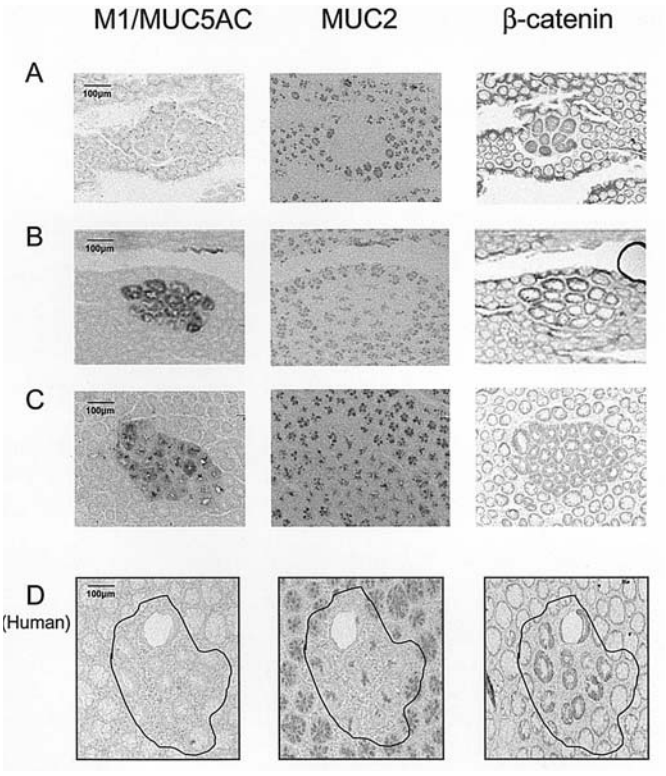

Figure 5. Rat (A-C) and human (D) BCAC. (A-C) Different patterns of expression of mucins observed in BCAC 36 weeks after DMH injection. MUC5AC, MUC2 and ß-catenin immunohistochemical staining of serial section in colon of rat exposed to DMH. Cytoplasmic localization of ß-catenin was evident in the lesion, while in adjacent crypts, ß-catenin was localized almost exclusively at the border of cells. (A) MDF lesion: a mucin-depleted focus with 10 crypts showing the lack of MUC2, and the near absence of M1/MUC5AC. After alcian blue immunohistochemistry, this lesion was well-characterized by a lack of staining (data not shown). (B and C) Non-MDF lesions: note that immunopositivity for M1/MUC5AC was detected in the whole epithelium of the lesion. The M1/MUC5AC immunopositive cells did not have the appearance of goblet cells. Adjacent normal crypts showed no immunostaining for gastric mucins. Color was developed with 3,3-diaminobenzidine (MUC5AC and ß-catenin) solution or VECTOR® SG substrate (MUC2). (D) Human MDF in *en face* preparation. The human colon tissue sample was taken from patients with colonic adenocarcinoma. Immunohistochemical staining was carried out with a rabbit polyclonal anti-human MUC2 antibody, a mixture of eight mouse monoclonal anti-human M1/MUC5AC antibodies (PM8) and a rabbit polyclonal anti-ß-catenin antibody. Color was only developed with 3,3-diaminobenzidine (DAB) solution.

described recently (13,14). The reduced expression of MUC2 in BCAC was associated with dysplasia (77% of these BCAC showed a dysplastic change). On the other hand, only 44% of all the BCAC population had dysplastic changes at the 36-week point.

We observed that in the BCAC, goblet cells were less numerous than in the HAF or healthy tissue. On serial sections, we demonstrated that these goblet cells were stained by alcian blue, whichever secreted mucin was expressed. In contrast, the 'colonocyte-like cells' which expressed MUC5AC were not revealed by alcian blue.

Mucin expression in human preneoplastic lesions. We then analyzed histological lesions (20 HAF and 20 BCAC) in human colons. As observed in rat, the crypt multiplicity was higher in BCAC than in HAF (14.6 vs 9). MUC5AC was detected in all HAF and the expression of MUC2 was not modified as compared to the adjacent mucosa. In contrast, MUC5AC was identified only in 76% of BCAC and MUC2 was decreased in 38.5% of these lesions. MDF were also detected in human colon (Fig. 5D).

Discussion

This is the first experimental study reporting a sequential analysis of the intestinal MUC2 mucin and of the gastric M1/MUC5AC mucin expression in histological lesions from the early phase of carcinogenesis in DMH-treated F344 rats. MUC2 is the main secretory mucin in the small intestine and in the colorectum, and is specifically produced by goblet cells. Several studies have shown by using *in situ* hybridization, Northern blot analysis or immunohistochemistry that the development of the majority of non-mucinous carcinomas of the colorectum was associated with a diminished expression of MUC2 (18-20,33-35). Recently, it was also demonstrated that MUC2 knockout mice develop colorectal tumors (21), thus suggesting that a decrease in MUC2 could contribute to colon carcinogenesis. In this context, the present study was undertaken in order to determine whether alteration of MUC2 occurred as early as the premalignant stages of colorectal carcinogenesis, and in which types of preneoplastic biomarkers and at which step of the transformation process these alterations become detectable. In the model of DMH-induced colon carcinogenesis, a decreased expression of the secreted mucin MUC2 was observed in very few HAF, whatever the degree of dysplasia, the crypt multiplicity or the week time-point. On the contrary, the proportion of BCAC which showed a reduction or a lack of MUC2 expression varied between 20 and 43% according to the time-point of the study. The observation that the histological lesions showing a reduced expression of MUC2 were mainly BCAC suggests a link between the activity of the Wnt pathway and the MUC2 gene. ß-catenin, a component of the cadherin-mediated cell adhesion system, is also a member of the APC/ß-catenin/TCF/Lef pathway (36). Alteration in the cellular localization of ß-catenin has been associated with a constitutive activation of the Wnt signaling pathway, leading to increased transcription of oncogenes such as *cyclin D1* or c-myc. Since these genes regulate cell growth, proliferation, differentiation, and transformation in the adult intestine, it is possible that the constitutive activation of Wnt pathway in BCAC may lead to the depletion of MUC2. In support of this idea, Blache *et al* demonstrated that the expression of the intestine transcription factor SOX9 that depends on the activity of the Wnt pathway was involved in the repression of the MUC2 gene (37). Another argument that can be advanced is that abrogation of the Wnt-signaling in colon cancer cell lines upregulated MUC2 (38). Taken together, these data suggest that alteration in the Wnt signaling present in the BCAC could be responsible for the reduction in the expression of MUC2 observed in these histological lesions.

BCAC have been reported as a new biomarker for rat colon carcinogenesis strongly predisposing to colon cancer (39,40). These lesions frequently contained mutations in the ß-catenin gene (Ctnnb1) that induce ß-catenin accumulation in the cytoplasm and nucleus (39,41-43). Alternatively, another

important mechanism underlying aberrant activation of the Wnt signaling pathway in carcinogenesis of the digestive organs and particularly in colorectal cancer is the silencing of the Wnt inhibitory factor-1 (WIF-1) due to promoter hypermethylation (44,45). In other cell systems, the influence of DNA methylation of CpG sites in the promoter region was also suggested as a possible regulatory mechanism of MUC2 gene expression (46) in pancreatic cell lines (47,48), in the gastric epithelium (49) and in human colorectal carcinoma cells *in vitro* and *in vivo* (19,50,51). Therefore, an attractive explanation for both the reduction in MUC2 expression and the accumulation of ß-catenin in the same histological lesions of DMH-treated rats, could be the hypermethylation of CpG sites in the promoter region of these genes. This hypothesis is supported by the observation that methylation of CpG islands was a molecular defect observed in human and rat ACF-like pre-neoplastic lesions (52,53). Pereira *et al* (54) also demonstrated that the estrogen receptor-α gene was hypermethylated in AOM-induced colon cancer (ACF), thus demonstrating that hypermethylation of specific CpG-rich promoter regions in tumor suppressor genes is a molecular alteration present in this rat model. This hypothesis remains to be tested.

During colonic carcinogenesis, alterations in the mucin expression pattern also include *de novo* expression of MUC5AC, a typical gastric mucin (25,26). MUC5AC is expressed in human colorectal adenocarcinomas (20) and in rat colon during 1,2-dimethyhydrazine or methyl-N'-nitroso-guanidine (MNNG)-induced carcinogenesis (23,25,26). In agreement with these data, our results showed that MUC5AC was aberrantly expressed before the formation of histological aberrant crypt and before disturbances in ß-catenin and in MUC2 expression. Indeed, two weeks after DMH-initiation, the expression of gastric mucins was apparent in some goblet cells of normal crypts. These crypts may be considered as precursors of HAF. The anti-rat gastric M1/MUC5AC mucin MAb then stained specifically goblet cells within HAF. We also demonstrated that, in HAF, the goblet cells that produced gastric mucins also expressed MUC2. The mechanisms underlying the capability of goblet cells to express MUC2 and MUC5AC genes simultaneously remain to be clarified. Nevertheless, it is known that these two genes share some sequence homology and have a clustered chromosomal location (15,16), raising the possibility that the expression of MUC2 and of MUC5AC genes may have common regulatory mechanisms. In agreement with this assumption, several studies have demonstrated that inflammatory mediators such as Il-1, TNF-α or COX-2 increased both MUC5AC and MUC2 expression in cell lines *in vitro* (55-57). Additionally, MUC5AC and TFF1, which are not normally detected in colonic tissue, were expressed in scattered colonic goblet cells, together with MUC2 in inflammatory bowel diseases (58,59). MUC5AC expression seems thus to be one of the many repair responses in colon epithelia. Interestingly, recent studies have demonstrated that the expression of enzymes associated with inflammation, such as inducible nitric oxide synthase and inducible COX-2, was increased in azoxymethane-induced rat colon carcinogenesis (43), thus suggesting that these enzymes could contribute to inducing *de novo* expression of MUC5AC in MUC2-producing goblet cells of HAF. However, the relationship between the expression of these enzymes and mucin localization remains to be determined.

In contrast, and with the exception of the MDF, we noted a temporal correlation between crypt multiplicity, *de novo* expression of MUC5AC and a decreased expression of MUC2 in the BCAC. In these histological lesions, the anti-rat gastric M1/MUC5AC mucin MAb also stained epithelial cells that were not phenotypically goblet cells. As the gastric mucin MUC5AC is expressed in the fetal colon (60), the presence of gastric mucins in BCAC could be regarded as a re-expression of oncofetal antigenicity. It is interesting to note that the different patterns of mucin expression observed in rat BCAC were similar to those detected in human BCAC in our study, but also in human colorectal cancer as described by Sylvester *et al* (20). These data suggest a link between BCAC and colon adenocarcinomas. Furthermore, these altered profiles of mucin observed in BCAC and in adenocarcinomas may also mean that the MDF, one of the categories of BCAC that are described in the literature as being the most probable cancer precursors (42), are not the only lesions to harbour a tumorigenic potential.

In conclusion, the results presented in this study clearly demonstrate that all of the HAF were characterized by a normal expression of MUC2 and by the presence of MUC5AC, this mucin being localized in goblet cells. In the BCAC, the time course analysis indicated that the expression of MUC5AC increased progressively with time. Mostly, this mucin may be localized in all the epithelium of the lesion. In the BCAC, the expression of the intestinal mucin MUC2 was also frequently decreased. This modification in the pattern of mucin expression in BCAC, as observed from the 8th to the 36th week, was related to the increase of crypt multiplicity and may thus correspond to the promotion step of colon carcinogenesis.

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