Histological and immunohistochemical observations of mucin-depleted foci (MDF) stained with Alcian blue, in rat colon carcinogenesis induced with 1,2-dimethylhydrazine dihydrochloride

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The usefulness of mucin-depleted foci (MDF), which have recently been proposed as a new preneoplastic biomarker in rat colon carcinogenesis, was histologically investigated in rat colonic tissues treated with 1,2-dimethylhydrazine dihydrochloride (DMH). The relationship among aberrant crypt foci (ACF), MDF and β-catenin accumulated crypts (BCAC) was examined by comparing the corresponding computer-captured images. Twelve male F344 rats were given DMH s.c. at a dose of 40 mg/kg body weight, once a week for 2 weeks, and randomly divided into two groups. Rats in group 1 were given normal drinking water, while those in group 2 were given drinking water containing indomethacin (IND) at 16 ppm for 6 weeks. All animals were sacrificed 8 weeks after the first DMH treatment. The resected colons were fixed in 10% formalin, and stained with Alcian blue for observation of ACF and MDF. Histological and immunohistochemical analysis revealed that the numbers of ACF, MDF and overlapping lesions in group 2 (treated with IND) were significantly decreased, compared with those in group 1. The number of BCAC in group 2 was also significantly lower than that in group 1. The reduction (61.5%) of MDF by IND was much greater than that (29.3%) of ACF. Analyses of the computer-captured images indicated that MDF had more frequent dysplastic changes and overexpression of β-catenin than did ACF. MDF having over 4 crypts or MDF with the appearance of ACF corresponded well to BCAC. These results suggest that MDF may be useful as an early biomarker in colon carcinogenesis. (Cancer Sci 2004; 95: 792–797)

Reliable biomarkers for colon carcinogenesis are needed for screening of chemicals for carcinogenic risk or potential chemopreventive efficacy on colon carcinogenesis. Aberrant crypt foci (ACF), which were originally described by Bird in unsectioned murine colon exposed to a colon-specific carcinogen, azoxymethane (AOM), have been recognized as early preneoplastic lesions.[1)] Subsequently, ACF induced by several kinds of chemical carcinogens have been widely used as a biomarker in short-term tests for prediction of colon carcinogenesis.[2)] However, some researchers have reported a lack of correlation between ACF induction and tumor development.[3–5)]

Previously, we reported β-catenin accumulated crypts (BCAC) as a new biomarker for rat colon carcinogenesis, strongly predisposing to colon cancer.[6, 7)] In addition to the accumulation of oncogenic β-catenin protein, the lesions harbored frequent β-catenin (Ctnnb1) gene mutations that are involved in the development of colon cancer.[8–12)] Histological observations of BCAC showed dysplasia, a hallmark of malignant potential, and increasing size with time after the carcinogen exposure.[7)] These observations indicate that BCAC are more likely to progress to malignant transformation than are ACF. However, their identification, based on immunohistochemical methods, is difficult in unsectioned colon and the procedure is complicated.

Recently, MDF (mucin-depleted foci) have been described to be preneoplastic lesions that can be used as a biomarker in colon carcinogenesis.[5, 13)] MDF are detected by using high-iron diamine Alcian blue pH 2.5 (HID-AB) staining. However, the relationship between MDF and BCAC is not clear. In the present study, we examined the identification and the characteristics of MDF by means of Alcian blue pH 2.5 (AB) staining, which is a convenient staining method, and we investigated the relationships among MDF, ACF and BCAC during rat colon carcinogenesis induced with a chemical carcinogen. We also examined the influence of indomethacin (IND), a non-steroidal inflammatory drug (NSAID) and chemopreventive agent.

Materials and Methods

Animals, diets and chemicals. Four-week-old male F344 rats were obtained from Japan SLC, Inc., Hamamatsu, Japan. All animals were housed in wire cages (3 rats/cage) with free access to drinking water and a basal diet, CE-2 (CLEA Japan, Inc., Tokyo), under controlled conditions of humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2°C). DMH (1,2-dimethylhydrazine dihydrochloride) and IND were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DMH was used to induce preneoplastic lesions, such as ACF and BCAC. Experimental drinking water was prepared on a weekly basis by dissolving IND at 16 ppm (w/v) in distilled water and stored in a cold room (4°C).

Experimental procedure. This study was approved by the animal welfare committee of our university. After quarantine for 1 week, 12 male F344 rats were divided into two groups. Starting at 5 weeks of age, rats in groups 1 and 2 (6 rats each) received DMH s.c. at a dose of 40 mg/kg body weight, once a week for 2 weeks. Rats in groups 1 and 2 were fed the CE-2 diet during the experiment. Rats in group 2 were supplied with drinking water containing IND at 16 ppm for 6 weeks, starting 1 week after the last injection of DMH. At 8 weeks after the first DMH treatment, all rats were sacrificed under CO_2 anesthesia. Immediately after sacrifice, the colon was removed, cut open along its longitudinal axis, attached flat on a paper filter, and then fixed in 10% buffered formalin.

Identification of ACF and MDF in unsectioned colon. AB staining was used to identify both ACF and MDF, instead of the conventional staining using 0.2% methylene blue. Briefly, the fixed

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Abbreviations: AB, Alcian blue pH 2.5; ACF, aberrant crypt foci; BCAC, β-catenin accumulated crypts; DF, dysplastic foci; MDF, mucin-depleted foci; DMH, 1,2-dimethylhydrazine dihydrochloride; HE, hematoxylin and eosin; HID-AB, high-iron diamine Alcian blue; IND, indomethacin; NLC, normal-like crypts.

colons were rinsed for 5 min in 3% acetic acid and then stained for 30 min with a solution of 1% AB pH 2.5 (Sigma Chemical Co., St. Louis, MO) in 3% acetic acid. These colons were rinsed for 10 min in 3% acetic acid to prevent nonspecific staining and then washed in distilled water. The stained mucosa including both ACF and MDF were photographed with a DP-50 digital camera (Olympus Optical Co., Ltd., Tokyo) and the positions of both lesions were marked on the captured images on a computer monitor (Fig. 1A). ACF was identified according to the following criteria: larger than, and elevated above, the adjacent normal crypts, with thickened cell walls lining the crypt and increased pericryptal area (Fig. 2, A and G), in accordance with other studies using methylene blue staining.[1, 14–16] We counted the number and multiplicity of ACF in each rat colon by counting the number of aberrant crypts (AC) forming each focus (AC/ACF). MDF was identified as focal lesions characterized by the absence or very small production of mucins (Fig. 2, D and G) under the light microscope at a low magnification, like ACF. The number and multiplicity of MDF per colon (number of crypt/MDF) were recorded. After counting both ACF and MDF, we used 0.2% methylene blue staining to confirm whether some lesions seen in Fig. 2, A and G were conventional ACF.

Histological and immunohistochemical examinations. After the identification of ACF and MDF, and storage of the images in a computer, all colons were cut in 2-cm intervals from the anal side and the colonic mucosa were embedded in paraffin for histological and immunohistochemical analyses. Colonic mucosal sections were examined by utilizing an *en face* preparation and 3 μm thick serial sections.[6, 7, 17] Sections were stained with hematoxylin and eosin (HE). The computer-captured images including ACF and MDF in unsectioned colons (Fig. 1A) were compared with the histological lesions in the sections (Fig. 1B). Three different types of crypt foci were noticed in HE-stained sections. The first type is histological ACF (Fig. 2B), which resemble macroscopic ACF and can be seen as lesions having larger crypts and wider lumens in the crypts as compared with the surrounding crypts. The second type is dysplastic foci (DF; Fig. 2, E and H), which were identified as focal lesions based on the following criteria: (a) nuclear stratification; (b) loss of nuclear polarity; (c) structural abnormality of the crypts; (d) Paneth cell metaplasia; (e) decrease or loss of goblet cells; (f) presence of mitosis. The third type is normal (or normal-like) crypts (NLC). The numbers of histological ACF, DF and NLC per colon were measured, and the ratios were also calculated.

Immunohistochemical analysis for β-catenin protein was also performed on each HE-stained serial section, using the universal immuno-enzyme polymer method (Simple Stain Rat MAX PO (M) kit; Nichirei Co., Ltd., Tokyo) with microwave accentuation. Briefly, after deparaffinization, sections were treated 3% hydrogen peroxide and 2% bovine serum albumin for 20 and 10 min, respectively. Then the sections were incubated with a primary antibody against β-catenin (Transduction Laboratories, Lexington, KY) for 1 h. For each case, negative controls were prepared on serial sections in the same way as described above, except that incubation with primary antibody was omitted. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 2 min. The lesions with immunopositivity in cytoplasm and/or nucleus were considered as BCAC (Fig. 2, C, F and I). The incidence (number of lesions per colon) and multiplicity (number of crypts per lesion) were measured and the positions of BCAC were marked

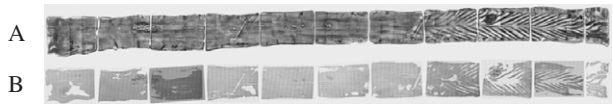

Fig. 1. Computer-captured images. A representative whole view of AB-stained mucosa of unsectioned colon (A) and the corresponding histological view (B).

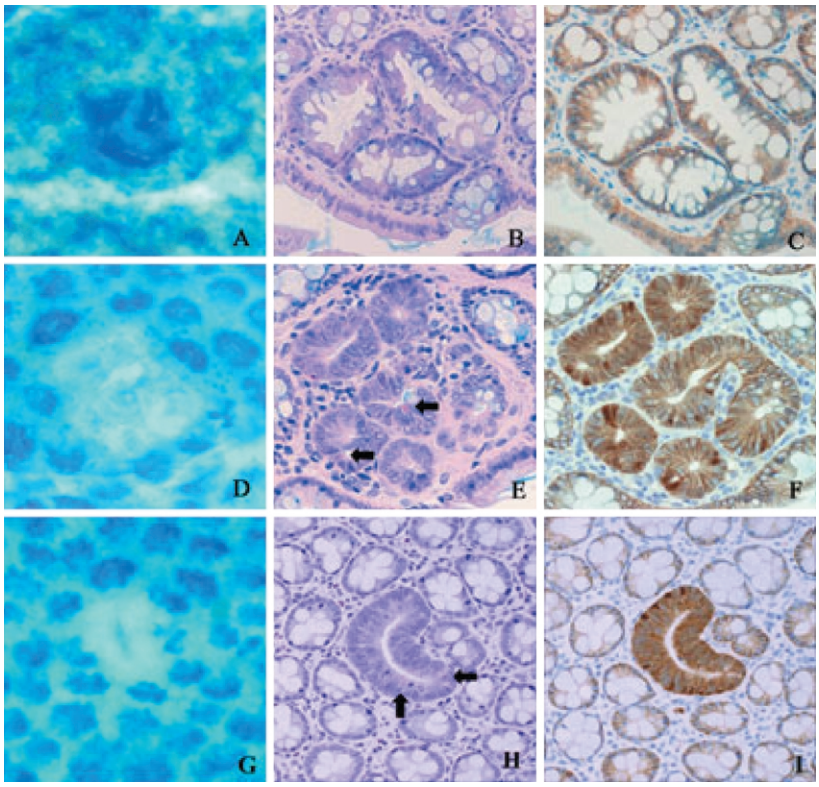

Fig. 2. The correspondence of the examined lesions in this study. A–C: ACF in AB staining. (A), the corresponding HE-stained lesion, histological ACF (B) and immunohistochemical β-catenin expression (C). D–F: MDF in AB staining. (D), the corresponding HE-stained lesion, DF (E) and β-catenin expression (F). G–I: overlapping lesion (single crypt) showing appearance of both ACF and MDF in AB staining. (G), the corresponding HE-stained lesion, DF (H) and the immunohistochemical β-catenin expression (I). ACF (A and G) show similar features to those observed in the conventional method, methylene blue staining. There are dysplastic changes with a loss of goblet cells and Paneth cell metaplasia (arrows) in E and H and strong β-catenin expression in cytoplasm and some nuclei in F and I, compared with those in B and C.

on computer-captured images in each section in the same way as descibed for the histological examination.

Estimation of the computer-captured lesions and statistical analyses. As mentioned above, the computer-captured images of ACF and MDF on the unsectioned materials were compared with the corresponding histological lesions and identified on a one-to-one basis geographically (Fig. 1). In addition, BCAC lesions were compared with the corresponding histological lesions by using the stored images. Data obtained in this study are presented as mean±SD. Student's *t* test, Welch's method, or the χ^2-test was used to determine the significance of differences between groups. *P* values of <0.05 were considered to be significant.

Results

General observations. All 12 F344 male rats survived to the end of the experiment, but none of them developed colon tumors. The mean values of the body weight, liver weight and relative liver weight in each group are shown in Table 1. They did not differ significantly between groups. There was no histologically apparent toxic effect in liver or kidney in rats of any group.

Identification of ACF and MDF. The numbers of ACF, MDF and overlapping lesions per rat are shown in Table 2. The number of ACF in group 1 was 150±23. Administration of 16 ppm IND (group 2) caused a significant reduction of ACF formation, to 106±23 ($P<0.01$). The number of ACF consisting of more than 4 aberrant crypts per rat in group 2 (21.7±10.6) was lower than that in group 1 (28.3±6.6), although this difference lacked statistical significance. The numbers of both MDF (7.5±1.9) and MDF consisting of more than 4 aberrant crypts (2.2±1.3) per rat in group 2 were significantly lower than those (19.5±5.2 and 7.5±3.9) in group 1 ($P<0.005$ and $P<0.05$), respectively. Interestingly, some overlapping lesions were seen (Fig. 2G). The incidence of the overlapping lesions corresponded to 1.5% of ACF and 11.1% of MDF in group 1 and 0.8% of ACF and 11.1% of MDF in group 2. Although the numbers of the overlapping lesions and lesions consisting of more than 4 aberrant crypts per rat in group 2 were lower than those in group 1, there was no statistically significant difference. As regarding the effects of IND, the reduction of MDF and the overlapping lesions was much greater than that of ACF (Table 2).

Comparison of the histological lesions corresponding to ACF and MDF. The colonic mucosal sections with *en face* preparation corresponded to approximately 84% of the unsectioned samples in this study. When the histological images were compared with the images of unsectioned colonic mucosa, the lesions corresponding to the ACF in groups 1 and 2 consisted of NLC, histological ACF and DF (Table 3), accounting for 8.0%, 84.3% and 7.7% in group 1 and 7.4%, 85.7% and 6.9% in group 2, respectively. The constituent ratios of the two groups were similar (no significant difference). On the other hand, the lesions corresponding to MDF consisted of NLC and DF, but no histo-

Table 1. Body, liver, and relative liver weights at experimental termination in each group

Group	Treatment (No. of rats)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	DMH alone (6)	281±20[1)]	10.2±1.2	3.60±0.22
2	DMH+16 ppm IND (6)	275±20	10.0±0.7	3.62±0.13

DMH, 1,2-dimethylhydrazine dihydrochloride; IND, indomethacin.
1) Mean±SD.

Table 2. The incidence of ACF, MDF and overlapping lesions in each group

Group	Total no. of ACF/colon/rat	No. of ACF containing of more than four ACs	Total no. of MDF/colon/rat	No. of MDF containing of more than four ACs	Total no. of the overlapping lesions /colon/rat	No. of overlapping lesions containing more than four ACs
1	150±23[1)]	28.3±6.6	19.5±5.2	7.5±3.9	2.17±1.6	0.67±0.5
2	106±23[2)] (29.3%)	21.7±10.6 (23.3%)	7.5±1.9[3)] (61.5%)	2.2±1.3[4)] (70.7%)	0.83±0.4 (61.8%)	0.17±0.4 (74.6%)

ACF, aberrant crypt foci; MDF, mucin-depleted foci; ACs, aberrant crypts. Values in parentheses show the reduction rate from group 1.
1) Mean±SD.
2) Significantly different from group 1 by Student's *t* test ($P<0.01$).
3, *4*) Significantly different from group 1 by Welch's *t* test. Superscripts: *3*) $P<0.005$ and *4*) $P<0.05$.

Table 3. The incidence of histological lesions corresponding to macroscopic appearance

Group	Lesions corresponding to ACF			Lesions corresponding to MDF			Lesions corresponding to overlapping lesions		
	No. of NLC	No. of H-ACF	No. of DF	No. of NLC	No. of DF	No. of DF	No. of NLC	No. of H-ACF	No. of DF
1	10.8±3.4[1)] (8.0%)	114±25 (84.3%)	10.5±3.7 (7.7%)	3.0±1.1 (21.4%)	0 (0%)	11.0±3.7 (78.6%)	0	0	1.33±1.0
2	7.2±2.2 (7.4%)	83±17[2)] (85.7%)	6.7±2.0 (6.9%)	2.0±0.6 (36.4%)	0 (0%)	3.5±0.8[3)] (63.6%[4)])	0	0	0.5±0.5

ACF, aberrant crypt foci; MDF, mucin-depleted foci; NLC, normal-like crypts; H-ACF, histological ACF; DF, dysplastic foci. Values in parentheses are the proportion in ACF or MDF.
1) Mean±SD.
2) Significantly different from group 1 by Student's *t* test ($P<0.05$).
3) Significantly different from group 1 by Welch's *t* test ($P<0.005$).
4) Significantly different from group 1 by χ^2-test ($P<0.05$).

logical ACF. NLC and DF accounted for 21.4% and 78.6% in group 1, and 36.4% and 63.6% in group 2, respectively. The incidence ratio of DF corresponding to MDF in group 2 was significantly lower than that in group 1 (χ^2-test, $P<0.05$). In addition, all of the overlapping lesions of both ACF and MDF were DF only (Fig. 2H).

Comparison of BCAC corresponding to unsectioned or histological lesions. The results are summarized in Table 4. The numbers of total BCAC and BCAC revealed as ACF or MDF in group 2 was significantly lower than those in group 1 ($P<0.001$, $P<0.001$ and $P<0.005$, respectively). Among the computer-captured images, 1.3% of ACF and 73.6% of MDF showed crypts revealed as BCAC in group 1. Further, 0.7% of the ACF and 63.6% of MDF in group 2 showed expression of β-catenin, and 75.2% of the overlapping lesions of both ACF and MDF were positive for β-catenin. Histologically, all of ACF and MDF showing the expression of β-catenin were revealed as DF, but not NLC or histological ACF. In addition, all MDF having over 4 crypts were positive for β-catenin in both groups (details not shown).

Discussion

In this study, we examined for the first time the relationship between MDF and conventional ACF, which have been considered as early preneoplastic lesions,[1)] on the unsectioned colon of DMH-treated rats, and we also investigated the relationship between the histological lesions and BCAC corresponding to each unsectioned lesion. The lesions found on DMH-treated colonic mucosa are summarized in Fig. 3. We found that 78.6% of MDF histologically showed dysplastic changes (we called these dysplastic foci), and 93.6% of these lesions showed accumulation of β-catenin (total 73.6% of MDF). On the other hand, 7.7% of ACF show dysplastic changes, and the lesions overexpressing β-catenin amount to only 16.2% of ACF with dysplastic lesions (total 1.3% of ACF). These ACF with dysplastic changes may coincide with dysplastic-type ACF, and our histological ACF seem to coincide with the hyperplastic type of ACF in previous reports,[18–20)] although the methods of identification were different. It was reported that β-catenin gene mutation was present only in dysplastic-type ACF.[18, 19)] Interestingly, the ACF with overexpression of β-catenin corresponded to only dysplastic foci in this study, and almost of them overlapped with MDF. Furthermore, as mentioned above, MDF showed much more frequent dysplastic change and β-catenin overexpression than ACF in this study. Previously, we reported that β-catenin gene mutation is more frequent in BCAC than in typical ACF, and this might indicate the potential to progress to malignant lesions.[6, 7)] Namely, the mutation of β-catenin gene seems to be necessary as a first step leading to preneoplastic lesions in rat colon carcinogenesis.[21, 22)] Therefore, MDF should be very useful as a biomarker in rat colon carcinogenesis, taking into account the overexpression or gene mutation of β-catenin as a preneoplastic marker. Overlapping lesions of ACF and MDF might be particularly important.

MDF detected with HID-AB in AOM-treated rodents was recently proposed as an additional biomarker in colon carcinogenesis.[5, 13)] However, while HID and AB (pH 2.5) stain sialomucins and sulfomucins, respectively, AB (pH 2.5) simultaneously stains both sialomucins and sulfomucins. Further,

Table 4. The incidence of BCAC and the characteristic histological findings in each group

Group	Total no.	Lesions corresponding to ACF			Lesions corresponding to MDF			Lesions corresponding to overlapping lesions			Others
		NLC	H-ACF	DF	NLC	H-ACF	DF	NLC	H-ACF	DF	
1	11.0±2.7[1)]	0	0	1.7±0.5	0	0	10.3±2.7	0	0	1.0±0.6	0
2	3.7±0.8[2)]	0	0	0.7±0.5[3)]	0	0	3.5±0.8[4)]	0	0	0.5±0.5	0
	(66.4%)			(58.8%)			(66.0%)			(50%)	

BCAC, β-catenin accumulated crypts; ACF, aberrant crypt foci; MDF, mucin-depleted foci; NLC, normal-like crypts; H-ACF, histological ACF; DF, dysplastic foci. Values in parentheses are the reduction rate from group 1.
1) Mean±SD.
2, *4*) Significantly different from group 1 by Welch's *t* test ($P<0.001$ and $P<0.005$, respectively).
3) Significantly different from group 1 by Student's *t* test ($P<0.001$).

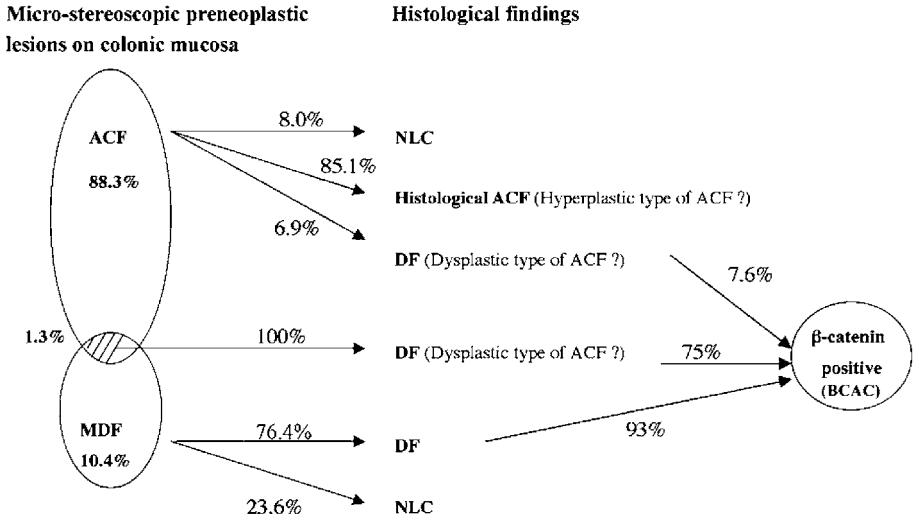

Fig. 3. Summary of the constitution of histological lesions. NLC, normal-like crypts; corresponding to ACF, MDF or the overlapping lesions in the colonic mucosa treated with DMH in this study. Descriptions in parentheses refer to preneoplastic lesions proposed in previous reports.[6, 18–20)]

since the detection of MDF by using HID-AB staining requires about 20 h, it is not practically convenient. The AB staining used in the present study can be done within 1 h. Therefore, we could detect both ACF and MDF on the same mucosa by using the AB simple staining. Moreover, it is not yet clear whether MDF and BCAC are identical lesions, to our knowledge. In this study, we found that MDF were identified by AB staining in unsectioned colons treated with DMH, and most of the MDF histologically demonstrated dysplastic features. Immunohistochemistry for β-catenin and distribution analysis using images indicated that almost all MDF corresponded to BCAC. The matching ratio between MDF consisting of more than 4 crypts and BCAC was higher than that of MDF consisting of 1–3 crypts. Therefore, it appears that MDF consisting of more than 4 crypts is a useful marker for detection of BCAC.

Mucins are highly glycosylated proteins that are the major components of the mucins that lubricate and protect the underlying intestinal epithelium.[23] Alterations of mucin expression and glycosylation have been observed in human colon cancer specimens,[24] but the role of these changes in tumorigenesis remains unclear. Moreover, a relative increase of sialomucins caused by a reduction of sulfomucins has been noted in colon cancer in human and rats.[25–29] It was also reported that the change in the phenotype of mucin was related to the degree of histological malignancy (dysplasia).[30] In Filipe's paper,[30] changes of mucous secretion, with predominance of sialomucins, were observed in the majority of the areas showing mild to moderate dysplasia, while the surrounding normal epithelium produced sulfomucins. In addition, it was mentioned that the mucous depletion was a common feature in areas of severe dysplasia and carcinoma. Therefore, the mucous change from sulfomucins to sialomucins might reflect early malignant transformation. A simple method for the identification of mucous depletion might be of great help in detecting early malignancy. Further information from proteomics investigations, including the factors underlying the changes of mucins, should be helpful to understand the process of carcinogenesis.

As regards the effect of IND, the result was similar to our previous findings in which the reduction rate of BCAC was higher than that of ACF in rat colons treated with celecoxib, a selective cyclooxygenase-2 inhibitor.[17] In addition, IND markedly reduced the number of DF corresponding to MDF as compared with that corresponding to ACF in this study. BCAC corresponded to 7.4% of all histological lesions derived from both ACF and MDF in DMH-induced colon, and almost BCAC are DF derived from MDF rather than ACF. These findings support the proposal[5, 13] that MDF might be a realistic preneoplastic biomarker, even in colon carcinogenesis induced by DMH. However, ACF is still important as a preneoplastic marker of colon cancer, because 1.3% of ACF (including the overlapping lesions with MDF) showed histological dysplastic change and the overexpression of β-catenin in DMH-induced colon, and the reduction rate of ACF with dysplastic change and β-catenin by IND was high (Table 4). Since there were some overlapping lesions of ACF and MDF, it should be examined whether ACF transform to MDF or *vice versa*. Furthermore, molecular investigations of MDF, such as β-catenin gene mutation studies, are needed.

In conclusion, we found that MDF resembling ACF in appearance and with more than 4 crypts were identical with BCAC, and the number of MDF was reduced by a chemopreventive agent. This MDF assay is simple, like ACF assay, and therefore, MDF may be useful as a biomarker of rat colon carcinogenesis.

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