Sequential Changes in Aberrant Crypt Foci and Lectin Expression in the Early and Late Stages of DMH-Induced Colon Carcinogenesis in Rats

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Background/Aims: The purpose of this study was to investigate the malignant potential of aberrant crypt foci (ACF) by measuring the multiplicity of crypts and lectin expression in the early and late stages of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis. Methods: Six-week-old Wistar rats were injected subcutaneously with DMH for 27 weeks. We classified ACF according to the number of crypts per ACF as a few crypts (≤3 crypts, FC ACF) or numerous crypts (≥4 crypts, NC ACF). Immunohistochemistry was used to evaluate lectin expression. Results: In the early stage, FC ACF (590/1,902, 31.0%) occurred more frequently than NC ACF (35/449, 7.8%); whereas in the late stage, NC ACF (176/449, 39.2%) occurred more frequently than FC ACF (324/1,902, 17.0%). The number of ACF peaked at 15 to 20 weeks. The ratio of NC/FC ACF increased gradually during carcinogenesis. The expression of both UEA1 and PNA was higher in NC ACF than FC ACF. Lectin expression increased in the late stage compared with the early stage. Conclusions: The expression of lectin was higher in NC ACF and ACF in the late stage. Therefore, ACF with higher multiplicities in the late stage may have more malignant potential in DMH-induced colon carcinogenesis. (Gut Liver 2012;6:229-234)

Key Words: Aberrant crypt foci; Ulex europaeus agglutinin-I; Peanut agglutinin; Colon carcinogenesis

INTRODUCTION

Aberrant crypt foci (ACF) are thought to represent putative preneoplastic lesions or intermediate markers of colon carcinogenesis in experimental animals and humans.1-3 ACF are described as lesions comprising enlarged crypts that are slightly elevated above the surrounding mucosa and whose crypts stain densely with methylene blue. ACF often have a slit-shaped luminal opening and an increased pericryptal space.2,4 In experimental animal models, colon carcinogens can induce various kinds of growth patterns, topographic features and numbers of ACF depending on the experimental protocol. ACF are highly heterogeneous in number and crypt multiplicity, and moreover, the sequential changes in ACF during carcinogenesis are debated.2,5-8

Many genetic markers including oncogenes such as K-ras and β-catenin have been used to show that ACF are precursors of colon cancer in animal studies.9,10 Fetal-type differentiation markers such as mucin and plant lectin reappear during cancer development, and are regarded as good markers of precancerous lesions.8,11 The outer surface of mammalian cells, including malignant cells contains a carbohydrate-rich coat, the glycocalyx. On the outside of the cell membrane, carbohydrate chains are covalently linked to lipids and proteins (so-called glycoconjugates). Glycoconjugates are involved in cell-to-cell and cell-to-matrix interactions, which are important during malignant progression. Glycoconjugate abnormalities are found in epithelial malignancies and precancerous lesions.12 These changes in membrane glycosylation can be detected histochemically with labeled lectins such as Ulex europaeus agglutinin-I (UEA1) and peanut agglutinin (PNA).11 However, there are few reports on lectin expression as a molecular marker of ACF.

The purpose of the current study was to evaluate the malignant potential of ACF by observing the morphological sequen-
tial changes in ACF and lectin expression in chemically induced colon carcinogenesis.

MATERIALS AND METHODS

1. Animal experiments

Six-week-old Wistar male rats weighing 100 to 120 g were used. The animals were handled according to the rules of laboratory animals at The Catholic University of Korea, Seoul, Korea. 1,2-dimethylhydrazine (DMH) (Sigma Chemicals, St. Louis, MO, USA) was used to induce experimental colon cancer. DMH was prepared as a 0.5% solution in 0.2 mM ethylene diamine tetra acetic acid (EDTA) and adjusted to pH 6.5 with sodium bicarbonate. DMH was injected subcutaneously at a dose of 20 mg/kg of body weight once a week for 27 weeks. Seven rats were sacrificed 5, 10, 15, 20, 25, and 30 weeks after the first DMH injection. As a normal control group, 6 rats were injected subcutaneously with EDTA/sodium bicarbonate according to the same schedule.

2. Detection of ACF

The colon was resected and washed gently with phosphate-buffered saline (PBS). The resected colon was cut open longitudinally, placed flat between filter papers, and fixed in 4% paraformaldehyde for 2 hours. Fixed colons were stained with 0.2% methylene blue, and the number of ACF was counted under a microscope. ACF were identified as lesions comprising enlarged crypts with an increased pericryptal area having a slightly elevated appearance above the surrounding mucosa and higher intensity staining by methylene blue. The tissue was then embedded in paraffin, sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E) (Fig. 1A). We classified ACF according to the number of crypts per ACF as few crypts (≤3 crypts, FC ACF) or numerous crypts (≥4 crypts, NC ACF).

3. Immunohistochemical analysis

Paraffin-embedded tissues including ACF were sectioned at a thickness of 4 μm and placed on poly-L-lysine-coated slides. Paraffin sections were deparaffinized in xylene and dehydrated in serial graded ethanol solutions. Endogenous alkaline phosphatase activity was blocked with 20% acetic acid. After rinsing in PBS, the sections were treated with blocking serum for 20 minutes, then incubated for 3 hours at room temperature with primary antibody PNA and UEA1 (diluted 1:50; Vector Laboratories Inc., Burlingame, CA, USA). After rinsing in PBS, the sections were incubated for 30 minutes at room temperature with an ABC-alkaline phosphatase kit (Vector Laboratories Inc.). Finally, the reaction products were visualized using Fast-Red (Vector Laboratories Inc.), counterstained with Mayer hematoxylin, and dehydrated, cleared, and mounted. The staining intensity of UEA1 was scored as follows: 0, <10 cells; 1+, 10 cells to 10% of goblet cells; 2+, 10% to 50% of goblet cells; and 3+, >50% of goblet cells (Fig. 1B–E). The staining intensity of PNA was scored as follows: 0, no staining of goblet cell mucus; 1+, individual goblet cells containing PNA-positive mucus; and 2+, individual glands containing numerous PNA-positive goblet cells (Fig. 1F–H).

4. Statistical analysis

The number of ACF is expressed as the mean and standard deviation. Conventional Student’s t-test were used to evaluate significant differences for continuous variables between two groups. All statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered statistically significant. Asterisk in the figures indicate significant differences between two groups.

Fig. 1. Ulex europaeus agglutinin-I (UEA1) and peanut agglutinin (PNA) expression determined by immunohistochemistry in aberrant crypt foci (ACF) (×200). (A) H&E staining of ACF. (B–E) Expression of UEA1 in ACF according to staining intensity (0, 1+, 2+ and 3+, respectively). (F–H) Expression of PNA in ACF according to staining intensity (0, 1+ and 2+, respectively).
RESULTS

1. Induction of ACF according to time sequence

In the experimental group, mucosal edema and hemorrhage became worse with repeated administration of DMH. Adenomas were observed at 20 weeks after the administration of DMH. Nonmucinous adenocarcinomas were first observed at 25 weeks, and mucinous adenocarcinomas developed at 30 weeks after the administration of DMH. In the control group, mucosal edema, hemorrhage, and atrophy were not observed, and there were no ACF or adenocarcinomas. The number of ACF stratified by crypt multiplicity according to time sequence are summarized in Table 1. During DMH-induced colon carcinogenesis, a total of 2,351 ACF developed; more were FC ACF (1,902) than NC ACF (449). In the early stage (5 to 10 weeks), FC ACF (590, 31.0%) were developed relatively more frequently than NC ACF (35, 7.8%). In the late stage (25 to 30 weeks), NC ACF (176, 39.2%) occurred more frequently than FC ACF (324, 17.0%) (p=0.006). The number of ACF was maximal at 15 to 20 weeks after the first carcinogen treatment, after which the number of ACF decreased gradually, irrespective of crypt multiplicity. However, the NC/FC ACF ratio increased progressively with time after DMH administration: 0 (0/278) at 5 weeks, 0.112 (35/312) at 10 weeks, 0.151 (76/502) at 15 weeks, 0.333 (162/486) at 20 weeks, 0.448 (117/261) at 25 weeks, and 0.936 (59/63) at 30 weeks (Fig. 2).

2. Expression of UEA1 and PNA in ACF, adenomas, and adenocarcinomas

UEA1 was not expressed in the colonic mucosa of the control group. PNA was weakly expressed in the Golgi apparatus of proximal colonic mucosal cells in some of the control group. Both UEA1 and PNA were overexpressed (score 2+ or 3+) in all adenomas and adenocarcinomas. The ratio of no staining (score 0) to strong staining intensity (UEA1 score 3+; PNA score 2+) ACF according to time sequence was shown in Fig. 3. The expression of UEA1 and PNA was progressively increased over time.

The expression of UEA1 increased in the late stage than in the early stage. In the early stage (5 to 10 weeks), UEA1 was expressed in the total ACF, FC ACF, and NC ACF as follows: staining intensity 0, 375 (60.0%); 370 (62.7%); 5 (14.3%); staining intensity 1+, 113 (18.1%); 106 (18.0%); 7 (20.0%); staining intensity 2+, 91 (14.6%); 70 (11.9%); 21 (60.0%); and staining intensity 3+, 46 (7.3%); 44 (7.4%); 2 (5.7%). The respective values in the late stage (25 to 30 weeks) were: staining intensity 0, 7 (1.4%); 7 (2.2%); 0; staining intensity 1+, 4 (0.8%); 4 (1.2%); 0; staining intensity 2+, 28 (5.6%); 25 (7.7%); 3 (1.7%); and staining intensity 3+, 462 (92.2%); 288 (88.9%); 174 (98.3%) (p<0.001, Fig. 4).

The expression of PNA also increased with time and in relation to the number of crypts. In the early stage (5 to 10 weeks), PNA was expressed in the total ACF, FC ACF, and NC ACF as follows: staining intensity 0, 541 (85.3%); 538 (89.8%); 3 (8.6%); staining intensity 1+, 74 (11.7%); 46 (7.7%); 28 (80.0%); and staining intensity 2+, 19 (3.0%); 15 (2.5%); 4 (11.4%). The respective values in the late stage (25 to 30 weeks) were: staining intensity 0, 205 (40.9%); 159 (49.1%); 46 (26.0%); staining

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Table 1. The Number of ACF according to Time Sequence in DMH-Induced Rat Colon Carcinogenesis

| Time, wk | ACF with ≤3 crypts | ACF with ≥4 crypts | p-value |
|----------|--------------------|--------------------|---------|
| 5        | 39.71±25.52        | 0                  | 0.006   |
| 10       | 44.57±15.75        | 5.00±2.58          | 0.001   |
| 15       | 71.71±25.14        | 10.86±6.60         | 0.001   |
| 20       | 68.57±24.51        | 21.57±13.97        | 0.001   |
| 25       | 37.28±14.99        | 13.86±5.79         | 0.005   |
| 30       | 9.00±6.90          | 8.42±3.64          | 0.851   |

Data are presented as mean±SD.
ACF, aberrant crypt foci; DMH, 1,2-dimethylhydrazine.

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Fig. 2. The ratio of numerous crypts (NC) aberrant crypt foci (ACF) to few crypts (FC) ACF according to the time sequence after 1,2-dimethylhydrazine administration.

Fig. 3. The ratio of no staining to strong staining intensity aberrant crypt foci (ACF) according to time sequence after 1,2-dimethylhydrazine administration.
UEA1, Ulex europaeus agglutinin-I; PNA, peanut agglutinin.
intensity 1+, 207 (41.3%), 114 (35.2%), 93 (52.5%); staining intensity 2+, 89 (17.8%), 51 (15.7%), 38 (21.5%) (p<0.001, Fig. 5).

DISCUSSION

In this study, using lectin expression, we intended to determine the malignant potential of ACF occurred during DMH-induced colon carcinogenesis. The DMH model of colon carcinogenesis is a valid, widely used model of experimental colon carcinogenesis. In most protocols of DMH-induced colon carcinogenesis, ACF or colonic tumors were confirmed at 8 to 12 weeks after application (short-term study) or at 40 weeks later (long-term study).

Therefore, we defined early stage as 5 to 10 weeks and late stage as 25 to 30 weeks, and compared the ACF in the early and late stage.

A total of 2,351 ACF with 35 adenocarcinomas developed within 30 weeks after DMH administration. FC ACF developed more frequently than NC ACF during carcinogenesis. FC ACF developed relatively more in the early stage compared to NC ACF, whereas vice versa in the late stage. These findings are consistent with the results of several previous reports showing that repeated administration of a carcinogen increases the number of ACF with multiple crypts. In our study, about a quarter of NC ACF occurred relatively early, at 10 to 15 weeks, but this proportion is not large compared to FC ACF occurred at the same time. ACF develop as early as 2 to 4 weeks after carcinogen administration, and ACF are a heterogeneous group of lesions. Most of ACF show a hyperplastic character, while only a small group of ACF may progress to ACF with dysplasia. NC ACF does not necessarily mean ACF with dysplasia, therefore, some of NC ACF will probably develop early.

Meanwhile, the number of NC ACF was maximal at 20 weeks...
after DMH injection and then decreased. It may be due to changes of some of ACF during the multistep process of colon carcinogenesis. We found colon adenoma first at 20 weeks, as in other reports. Some selected ACF may progress to adenoma or adenocarcinoma. Also, some ACF may be regressed by apoptosis or restored to the normal crypts during the process. Therefore, the number of ACF can be reduced in the late stage.

We assessed the NC/FC ACF ratio according to time sequence. It tended to increase gradually through the course of carcinogenesis. These results suggest that the number of crypts in the ACF is a more important factor for identifying the development of malignancy than the total number of ACF. It was consistent with previous findings. Further studies are warranted to verify the NC/FC ACF ratio as a predictive marker of colon malignancy.

We measured the lectin expression of ACF to evaluate their malignant potential because the lectin–binding capacity to a cell is a well-known marker of malignancy. Lectins are carbohydrate–binding proteins of nonimmune origin that agglutinate cells and/or precipitate polysaccharides or glycoconjugates. Lectin UEA1 is a combined glycoprotein containing α-1 fucosyl residue, which binds to neoplastic cells but is incapable of binding to cell surface glycoconjugates in most nonneoplastic mucosal cells. In breast cancer, tumors with binding sites for UEA1 are associated with poor prognosis. In the present study, the expression of UEA1 was higher in NC ACF than in FC ACF. Therefore, ACF with high multiplicity have a higher capacity for malignant progression than do ACF with low multiplicity. The expression of UEA1 was also higher in ACF appearing in the late stage than those appearing in the early stage, irrespective of ACF multiplicity. These findings suggest that ACF found in the late stage may be more likely to progress into malignant lesions than ACF found in the early stage.

Alterations in cell surface carbohydrate on colon cancer cells include neoexpression of O-linked core carbohydrate antigens. The typical antigen is the Thomsen-Friedenreich (TF) oncofetal carbohydrate antigen of O-linked oligosaccharide chains on glycoproteins. This antigen is expressed in the neonatal colon but is normally masked by further glycosylation or sulfation in the adult colon. The expression of TF antigen increases in hyperplastic and adenomatous colonic polyps, and in colon cancer. TF antigen is detected by the lectin PNA, suggesting that PNA together with UEA1 might be useful molecular markers for predicting tumorigenic potential. In our study, the expression of both UEA1 and PNA was higher in NC ACF than in FC ACF, and in ACF found in the late stage than in the early stage.

In conclusion, lectin expression, including UEA1 and PNA, was higher in NC ACF and ACF in the late stage. These findings suggest that either ACF constituting a number of crypts or ACF found in a later stage may have higher malignant potential in DMH-induced colon carcinogenesis. Therefore, ACF with higher crypt multiplicities may be considered to be a valid biomarker in colon carcinogenesis.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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