RESEARCH COMMUNICATION

Effect of 2-(carboxyphenyl) Retinamide and Genistein on the Formation of Early Lesions in 1,2-dimethylhydrazine-induced Colon Carcinogenesis in Rats

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Abstract

Aberrant crypt foci (ACF) are recognized as preneoplastic lesions for colon cancer, and ACF in rodents are widely used as an intermediate biomarker to predict tumorigenicity in the colon. However, a lack of correlations between the formation of ACF and the development of colonic tumors has been reported in several studies. For example, 2-(carboxyphenyl) retinamide (2-CPR) and genistein were reported to inhibit the carcinogen-induced formation of ACF, whereas both of them were later found to enhance colon tumorigenesis in rats treated with azoxymethane (AOM). Recently, we have identified β-catenin-accumulated crypts (BCAC) in the colon of rats shortly after administration of AOM, and provided evidence that these are independent early lesions of classical ACF, and BCAC might be direct precursors for colon cancers. In the present study, we performed a comparative analysis of the modifying effects of 2-CPR and genistein on 1,2-dimethylhydrazine (DMH)-induced BCAC and ACF in male F344 rats. Dietary administration of 2-CPR (315 ppm) significantly reduced the total number, multiplicity and size of ACF in DMH-exposed colonic mucosa, while genistein (250 ppm) had no significant effects on DMH-induced ACF formation. In contrast, both of 2-CPR and genistein significantly enhanced the multiplicity and size of DMH-induced BCAC when compared with DMH alone group. In addition, both 2-CPR and genistein significantly increased the proliferating cell nuclear antigen (PCNA) index preferentially in BCAC. Together with previous findings that 2-CPR and genistein are tumor promoters in the colon, our results support the concept that BCAC are precursors of colon tumors and suggest that these lesions are more reliable short-term biomarkers for colon carcinogenesis in rodents than ACF.

Key words: Myricitrin - beta-catenin accumulated crypts - aberrant crypt foci – prevention - colon cancer.

Introduction

Colon cancer is one of the leading causes of cancer morbidity and mortality in the world. This malignancy has been regarded as one of the most preventable forms of visceral cancers, and a number of studies for its chemoprevention have been attempted (Reddy, et al., 2005). To detect early appearing lesions in the colorectal carcinogenesis is particularly important, since such cell-population can be useful for a reliable surrogate biomarker on chemoprevention of large bowel cancers.

Aberrant crypt foci (ACF) were initially identified topographically by Bird on the colonic mucosa of rodents exposed to colon-specific carcinogens (Bird, 1987). ACF were defined as crypts that: have altered luminal openings; exhibit thickening of the epithelium; and are larger than adjacent normal crypts (Bird et al., 1989). Several other colon-specific carcinogens including 1,2-dimethyl-hydrazine (DMH) were also shown to induce ACF (McLellan, et al., 1988). To date, ACF have been accepted as possible preneoplatic or precancerous lesions of colorectal cancers. A number of pathological and biochemical characteristics of rodent ACF have been identified. Increased expression of c-fos and cyclin D1 were associated with ACF (Otori et al., 1999, Stopera et al., 1993, Stopera et al., 1992), and frequent K-ras gene mutation has also been found in ACF (Stopera, et al., 1992, Tachino et al., 1995). Although rodent ACF have been used as biomarkers for screening effective chemopreventive agents or for risk assessment of chemicals in the environment (Kawabata, et al., 2000, Yoshimi, et al., 1997), several previous studies have revealed a lack of correlations or discrepancy between tumor development and induction of ACF (Magnuson et al., 1993, Pereira et al., 1994, Rao et al., 1997, Zheng et al., 1999).

Recently, by using en face preparations of the colonic mucosa of rats exposed to colon specific carcinogen, we identified "β-catenin accumulated crypts (BCAC)" with a

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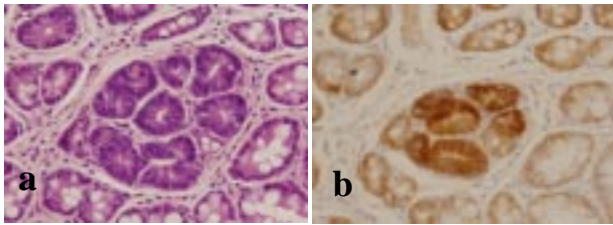
Figure 1. Appearance of a BCAC. a) H&E, b) β-catenin staining

number of different morphological properties from those of classical ACF (Figure 1) (Mori, et al., 2004, Yamada, et al., 2000, Yamada, et al., 2001). BCAC reveal histological dysplasia and are frequently accompanied by Paneth cells (Yamada et al., 2001) (see Figure 1). Importantly, β-catenin gene mutations, which are observed in most colon tumors in rats (Takahashi, et al., 1998), were also found frequently in BCAC (Yamada et al., 2003). The number of the crypts/lesion and histological abnormality of BCAC significantly increase with time (Yamada et al., 2001). Interestingly, frequency of β-catenin mutations was higher in BCAC than that of ACF and cell proliferative activity in BCAC is also higher than ACF. Furthermore, a selective inhibitor of cyclooxygenase-2, a promising chemopreventive agent against colon carcinogenesis, also suppressed the formation of BCAC in AOM-treated rats, and the effect is stronger than that in ACF (Yamada et al., 2001). These findings suggest that BCAC are more likely to be direct precursors for colon cancers.

The purpose of the present study was to assess whether the colonic BCAC are sensitive and reliable short-term biomarkers for colon carcinogenesis. Here we examined the modulating effects of 2-CPR and genistein, which were reported to inhibit ACF formation and were also found to
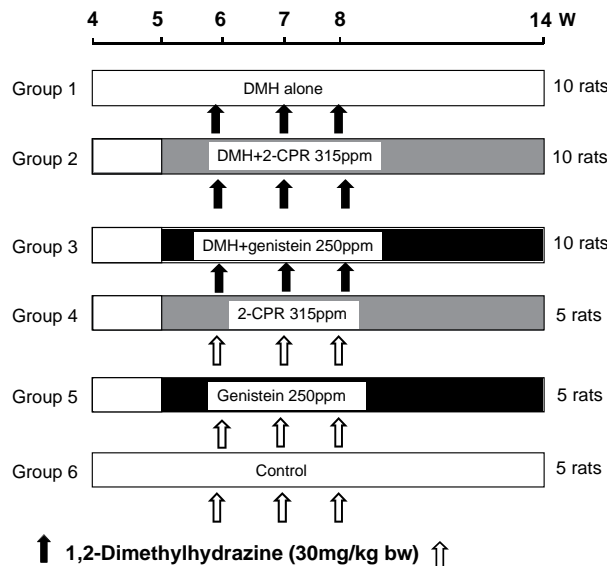
enhance colon tumorigenesis (Pereira et al., 1994; Rao et al., 1997; Zheng et al., 1999), on the formation of DMH-induced ACF and BCAC in the colon of rats. To detect mode of actions of these modifying agents, we also evaluated the proliferating cell nuclear antigen (PCNA) index to assess whether 2-CPR or genistein affects cell proliferation activity in such early lesions.

Materials and Methods

Animals, diets and chemicals

Four-week-old male F344 rats were obtained from Japan SLC Inc., Hamamatsu, Japan. DMH, 2-CPR and genistein were purchased from Sigma-Aldrich Chemical Co., NARD Institute Ltd.(Japan), and TCI (Tokyo), respectively. All experimental diets were formulated based on the composition of AIN-76A diet (CLEA Japan, Tokyo). Animals were housed to wire cages (2-4/cage) in an animal holding room under controlled conditions with $23\pm2°C$, $50\pm10\%$ humidity, and 12h light/dark cycles. They were allowed ad libitum access to AIN-76A control diet and water.

Experimental procedure

Four-week-old male F344 rats were divided into 6 groups (Figure 2). Beginning at 5 weeks of age, rats in groups 2 (n=10) and 4 (n=5) were fed the AIN-76A diet containing 2-CPR (315 ppm) and continued on this diet until the end of the experiment. Rats in groups 3 (n=10) and 5 (n=5) were given genistein (250 ppm) diet throughout the experiment. The other groups were fed with the control diet. Beginning at 6 weeks of age, rats in groups 1 (n=10), 2, and 3 received DMH s.c. injection at a dose of 30 mg/kg body weight, once a week for three consecutive weeks. Rats in groups 4, 5, and 6 (n=5) intended for vehicle treatment received the same volume of saline. At 14 weeks of age, all animals were killed with ether anesthesia. Their colons were removed, slit open longitudinally, and washed with phosphate-buffered saline (PBS). Then, they were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 h at 4°C for ACF and BCAC analysis.

Identification of ACF and BCAC

All colons were stained with methylene blue for determining the frequency, multiplicity and size of ACF under a light microscope. ACF were recorded according to standard procedures that are routinely used in our laboratory. The criteria used to identify ACF were as follows: they are larger and elevated above the adjacent normal crypts with thickened cell walls lining the crypt and increased pericryptal area. For measurement of diameters of ACF, colons from three rats in each group were selected randomly and evaluated. After removing the rectal sides (1 cm from the anus), all colons were then cut into four segments (distal, medium-distal, proximal-medium and proximal) with equal length (~3cm each). The distal, medium-distal, and proximal-medium segments from all the colons were examined for identification of BCAC in the present study. They were embedded in paraffin blocks utilizing en face preparation and processed by conventional histological methods. Serial sections with

Figure 2. Experimental protocol. Rats were divided into 6 groups. Beginning at 5 weeks of age, the basal dietary with and without 2-CPR (315 ppm) or genistein (250 ppm) was started and continued until end of the experiment. After a week, rats in groups 1, 2, and 3 received DMH s.c. at a dose rate of 30 mg/kg body wt, once a week for 3 successive weeks. The left groups intended for vehicle treatment received the same volume of saline. At 14 weeks of age, all animals were killed, then their colons were prepared for ACF and BCAC analysis.

4 μm thickness from a total of 135 segments (30, 30, 30, 15, 15 and 15 segments from groups 1, 2, 3, 4, 5 and 6, respectively) were used. Two and four sections from the 10 serial sections of each colonic segment were selected for immunohistochemistry of β-catenin and hematoxylin and eosin staining, respectively. The labeled streptavidin-biotin method using an LSAB kit (Dako, Glostrup, Denmark) with microwave accentuation was performed for immunohistochemical analysis.

After deparaffinization, sections were treated with 3% hydrogen peroxide for the 10 min and incubated with 2% bovine serum albumin for 30 min. Then the sections were incubated with a primary antibody against beta-catenin (1:1000, Transduction Laboratories, Lexington, KY). Negative control using the serial section was prepared in the same manner but the primary antibody omitted. Horseradish peroxidase activity was visualized by treatment with hydrogen peroxide and diaminobenzidine for 5 min. In addition to histological abnormality, the lesions with increased immunopositivity in the cytoplasm and /or nucleus were considered as BCAC. As observed previously, ACF showed little accumulation of beta-catenin, less nuclear atypia than BCAC, and no metaplastic change was observed. The frequency (number of lesions/cm2), multiplicity (number of crypts/lesion) and size (average of the longest and shortest diameters) were measured on each section.

Immunohistochemistry of proliferating cell nuclear antigen (PCNA)

Immunohistochemical staining for PCNA was performed by the streptavidin-biotin method as described above (Dako). The sections were incubated with an anti-PCNA antibody (monoclonal mouse anti-Proliferating cell Nuclear Antigen; Dako; 1:100 dilution). For determination of PCNA-positive index, 7~25 crypts in each lesion (ACF, BCAC) were examined. The number of PCNA positively stained nuclei in each crypt was recorded. The PCNA positive index (number of positive stained nuclei x 100/total number of nuclei counted) was then calculated.

Statistical analysis

Results of BCAC and ACF were analyzed statistically by Students't test or Welch' t test. P values of <0.05 were considered significant.

Results

General observation

The average body weights of rats in groups 1-6 at the end of the experiment were 270 ± 19, 254 ± 10, 261 ± 13, 254 ± 14, 272 ± 24 and 268 ± 30 grams (meanÅ}SD), respectively. The body weights of animals fed the 2-CPR diet (group 2 and 4) were significantly less than that of control diet group (group 6). However, the lower body weights did not relate to BCAC induction, as the rats treated with 2-CPR but without DMH (group 4) were not found to develop BCAC in their colons. There were no gross abnormalities or signs of toxicity observed in any of the groups.

ACF data

To evaluate the effects of dietary 2-CPR and genistein on the DMH-induced ACF formation, we first examined ACF on the surface of unsectioned colonic mucosa with the conventional methods that are routinely used in our laboratory. Data for ACF are summarized in Table 1. Administration of 2-CPR in the diet (group 2) significantly reduced the total number, multiplicity and size of DMH-induced ACF as compared with the control diet (group 1). However, treatment with genistein (group 3) had no significant effects on DMH-induced ACF formation. In the saline-treated groups (groups 4, 5 and 6), there was no evidence of ACF formation. As shown in Figure 3, the 2-CPR diet significantly inhibited the development of large ACF containing >4 aberrant crypts/focus.

BCAC data

Data are summarized in Table 2. Exposure to 2-CPR or genistein in the diet (groups 2 or 3) significantly increased crypt multiplicity and the sizes were also significantly larger in rats fed 2-CPR and genistein than that in rats with control diet. As shown in Figure 4, the numbers of large BCAC with ≥7 crypts/lesion were significantly increased in rats fed the 2-CPR or genistein diet as compared with those fed the control diet (group 1). We observed a tendency to increase the number of BCAC by 2-CPR or genistein, however, there was no significant difference. These results indicate that the 2-CPR or genistein diet significantly enhanced the development of
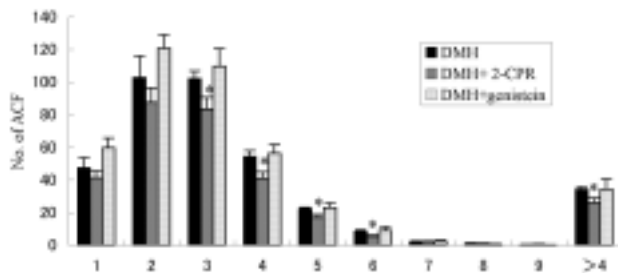
Table 1. Effects of dietary 2-CPR and Genistein on the Frequency, Multiplicity and Sizes of ACF

	Control	2-CPR	Genistein
Frequency (per colon)	353.3 ± 47.6[a] (10)[#]	284.4 ± 65.7[b] (10)	381.4 ± 92.6 (10)
Multiplicity	2.84 ± 1.30 (3276)	2.76 ± 1.31[b] (2789)	2.86 ± 1.51 (3810)
Size (diameter,μm)	197.8 ± 70.7 (349)	183.3 ± 66.3[c] (292)	192.9 ± 63.6 (182)

[a]Mean±SD [b,c]Significantly different from control diet group by Student's t test at p<0.05, p<0.005 [#]Data in brackets are for numbers of animals or lesions

Table 2. Effects of dietary 2-CPR and Genistein on the Frequency, Multiplicity and Sizes of BCAC

	Control	2-CPR	Genistein
Frequency (per cm²)	2.05 ± 0.70[a] (7)[#]	2.17 ± 1.30 (7)	2.37 ± 0.79 (7)
Multiplicity	2.76 ± 2.00 (136)	3.38 ± 2.52[b] (145)	3.37 ± 2.56[b] (156)
Size (diameter,μm)	85.9 ± 32.5 (136)	94.5 ± 37.9[b] (145)	100 ± 45.2[c] (156)

[a]Mean±SD [b,c]Significantly different from control diet group by Welch t test at p<0.05, p<0.005 [#]Data in brackets are for numbers of animals or lesions

Figure 3. Effects of Dietary 2-CPR or Genistein on the Multiplicity of ACF. Error bars represent SD. * p<0.05 vs. DMH alone

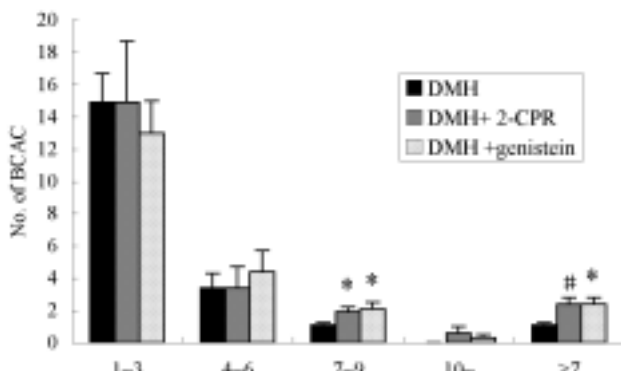**Figure 4. Effects of Dietary 2-CPR or Genistein on the Multiplicity of BCAC**. Error bars represent SD. * p<0.05 #p<0.01 vs. the DMH alone
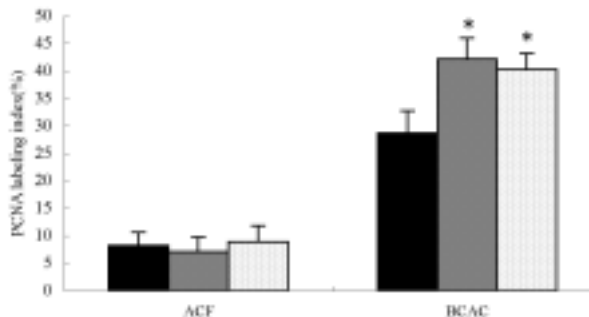

Figure 5. PCNA-labeling indices in ACF and BCAC. Error bars represent SD. * p<0.05 vs. DMH alone

BCAC, but not ACF, suggesting that the expression of BCAC is more related to the tumor promoting effects of 2-CPR and genistein.

PCNA-labeling indices of ACF and BCAC

The PCNA-labeling indices in ACF and BCAC were shown in Figure 5. The mean PCNA-labeling indices in BCAC of group 2 and 3 were significantly higher than that of group 1, while those in ACF have no significant differences between group 1 and groups 2 or 3.

Discussion

ACF, like an enzyme-altered foci in rodent hepatocarcinogenesis (Ito, et al., 1992), have come to be generally accepted as putative preneoplatic or precancerous lesions for colorectal cancers (Bird, 1995). ACF have been proposed as a biomarker for short-term screening assay for potential carcinogens and chemopreventive agents of colon cancer (McLellan et al., 1988; Pereira, et al., 1991).

Nevertheless, some data have given an evidence of a lack of correlations between tumor development and induction of ACF (Rao et al., 1997; Zheng et al., 1999). Such data also imply the possibility that ACF analysis generated some false positive or negative results in the screening assay. Previous studies have reported that 2-CPR and genistein, which have very potent effects in preventing the formation of ACF, enhanced, rather than prevent, colon tumorigenesis in rats treated with AOM (Rao et al., 1997; Zheng et al., 1999, Zheng, et al., 1997). In this study, we investigated the modulatory effects of dietary 2-CPR or genistein on the formation and development of two types of colonic early lesions, BCAC and ACF by use of DMH-induced rat colon carcinogenesis model. Consistent with previous findings, administration of 2-CPR significantly reduced the total number, the multiplicity and the size of ACF as compared with those with the control diet. In contrast, we could not detect any effect of genistein on ACF expression. It is noteworthy that both of 2-CPR and genistein significantly increased crypt multiplicity and size of BCAC. Together with the previous findings that 2-CPR and genistein acted as tumor promoters in the colon, our results suggest that BCAC are a reliable short-term biomarker for colon carcinogenesis in rodents. Present findings are supported by our recent findings that cholic acid, a typical tumor-enhancing chemicals, was found to decrease formation of ACF in rats, but it promoted the development of AOM-induced BCAC (Hirose et al., 2003).

The precise mechanisms in which 2-CPR has opposing effects on the formation of ACF and BCAC are not clear. In humans and rodents, ACF harbor frequent K-ras mutations (Pretlow et al., 2005), and mouse strains carrying oncogenic alleles of K-ras develop ACF in the colon (Johnson et al., 2001). Meanwhile, frequent b-catenin mutations are detectable in rat BCAC (Yamada et al., 2003), and ApcMin/+ mouse, which has mutation at the Apc gene, develops a number of BCAC in the colon (Yamada et al., 2002; 2003). The findings imply that the K-ras activation is associated with the formation of ACF, whereas the activation of b-catenin signaling pathway is involved in the formation of BCAC. Suppressing effects of 2-CPR on the formation of ACF observed in this study may be attributable to inhibitory effect of 2-CPR on the K-ras activation. Indeed, a recent study also suggests that Ras signaling pathway is one of the chemopreventive targets of retinoids (Tsai et al., 2006). It is interesting to note that ApcMin/+ mice develop numerous intestinal tumors as well as BCAC, whereas activated K-ras mice develop only ACF but no tumors in their colon, suggesting that BCAC are direct precursors for colon tumors. Our findings that 2-CPR or genistein, which have been reported to be tumor-promoters in the colon, enhanced the growth of BCAC, support the notion that BCAC are direct precursors of colon tumors.

The fact that administration of 2-CPR or genistein promoted the growth of BCAC, but not the incidence of BCAC suggests that they act in the promotion phase rather than initiation phase. Cell proliferation has long been known to play a significant role in the promotion and progression stage of carcinogenesis. In addition, PCNA index in colonic crypts is regarded as a putative intermediate marker for colon cancer risk (Yamada et al.,

1992) and many chemopreventive agents exert their inhibitory action through reduction of the cell proliferating activity in the target tissues (Kawabata et al., 1999, Yoshimi et al., 1999). In this study, PCNA-labeling index was increased in DMH-induced BCAC, but not in ACF by administration of tumor promoting 2-CPR or genistein. The results suggest that increased proliferative activity of cells initiated with a carcinogen might account for promoting effects of 2-CPR and genistein.

In conclusion, this study reports the opposing effects of dietary 2-CPR and genistein on the formation of two different types of early lesions of colon carcinogenesis. Dietary 2-CPR and genistein, tumor-enhancing agents, promote the formation of BCAC, supporting the proposition that BCAC are not only precursors of colon cancers but also a suitable short-term biomarker for colon carcinogenesis.

Acknowledgements

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