SHORT COMMUNICATION

Growth features of aberrant crypt foci that resist modulation by cholic acid

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Previously, we demonstrated that feeding rats a diet containing 0.2% cholic acid (CHA-diet) resulted in the elimination or remodeling of a number of aberrant crypt foci (ACF) in their primal stages (1–3 crypts/focus). The present investigation was conducted to determine if ACF with advanced growth features will respond differently than their primal counterparts to the CHA-diet. Sprague–Dawley male rats were injected with azoxymethane (20 mg/kg) and maintained on a control diet for 21 weeks. At week 21, three rats were killed and their colons were assessed for ACF. The remaining animals were randomly divided into two groups, which were fed a control diet or a CHA-diet respectively. After 3 weeks of feeding, the rats (n = 5) were killed and their colons were assessed for the number, size (area occupied by each focus) and crypt multiplicity (number of crypts/focus). The CHA-diet resulted in a significant (P < 0.05) reduction in the number of ACF with 1–2 crypt multiplicity. When compared to the control group, the CHA group ACF with 1–2 crypts/focus were reduced by 70%; with 3–4 crypts/focus, a 48% reduction; and with >4 crypts/focus, a 50.4% reduction. Treatment with CHA resulted in a marked reduction in the population of ACF with 1–2 or 3–4 crypt multiplicity with area >5–10×10^-2 mm^2. These findings demonstrate that ACF with advanced growth features are phenotypically different from their primal counterparts in resisting the responses elicited by the CHA-diet even at a late time point, and that morphological heterogeneity among ACF may represent different biological states.

Aberrant crypt foci (ACFs) are purported preneoplastic lesions of colon cancer (1–3). Phenotypic and genotypic features of ACF support this contention and this has been reviewed recently (4). Aberrant crypt foci are also present in human colons and display morphologic and proliferative features similar to those found in rodent colons (5–8). If indeed ACF are precursor lesions of colon cancer as suggested, the effect of modulators of colon carcinogenesis on the morphologic, growth and genotypic features of ACF, may provide important insight into the pathogenesis of colon cancer and the value of ACF in this process.

The features of ACF which could be easily evaluated by topographic assessment of the whole colon are their number, distribution along the length of the colon, and size (area occupied by each ACF) and the number of crypts constituting each focus (crypt multiplicity). The number and crypt multiplicity of ACF have been utilized by researchers to assess and identify potential modulators of colon carcinogenesis. Previously we reported that rats fed a diet containing 0.2% cholic acid by weight (CHA-diet) had a lower total number of ACF in their colons (9,10) than those fed a 0% cholic acid diet (control diet). These findings were interesting and considered important in light of the fact that in previous studies rats fed the CHA-diet concurrent to carcinogen administration, had enhanced tumour incidence compared to those that were fed a control diet (11,12), and we have proposed that ACF are preneoplastic lesions. Subsequent studies revealed that the CHA-diet was able to inhibit the development of a majority of ACF and eliminate, or remodel, a select population of ACF that had developed in the rat colons 4 weeks following a single injection of azoxymethane (10). The ACF that were eliminated or remodelled by CHA feeding (10) had a crypt multiplicity of 1–3, whereas those with a crypt multiplicity of ≥4 did not appear to be affected by the CHA-diet. However, the number of ACF with crypt multiplicity >4 was too small (1–2/colon) to establish if these ACF were indeed resistant to elimination or remodelling by the CHA-diet (10). In the present study, we utilized the CHA-diet to gain further insight into the biological heterogeneity of ACF. In particular, we investigated whether ACF with different growth features and that was well established in the colon would respond differently to the modulating effect of the CHA-diet. In this report, we demonstrate that ACF with higher crypt multiplicity resists elimination or remodelling by CHA-diet. Most importantly, the findings suggest that morphological heterogeneity among ACF could be useful in assessing their biological states.

Male weanling Sprague-Dawley rats were obtained from Campus Breeding Facility, University of Manitoba (Winnipeg, Canada). Animals were housed in wire cages with a 12 h light/dark cycle. Temperature and humidity were controlled at 22°C and 50% respectively. Upon receipt, animals were allowed to acclimatize for 1 week and were given the laboratory chow. The carcinogen azoxymethane (AOM) (Sigma Chemical Co., St Louis, MO), was diluted in sterile saline. Animals received a single s.c. injection of AOM (20 mg/kg body weight) or sterile saline.

Diets were formulated based on the composition of the AIN-76 diet as described previously (9) with the exception that dextrose replaced sucrose as a source of carbohydrate. The control diet (CON) contained 200 g vitamin free casein, 3 g DL-methionine, 500 g dextrose, 150 g cornstarch, 50 g corn-oil, 50 g cellulose, 10 g AIN-76 vitamin mix, 35 g AIN-76 mineral mix and 2 g choline mitartrate per kg diet. The CHA-diet was the AIN-76 diet plus 0.2% cholic acid (Sigma Chemical, St Louis, MO) by weight.

The experimental protocol is presented in Figure 1. Following acclimatization, all animals (Sprague–Dawley male rats) received a single injection of AOM (20 mg/kg) and were maintained on a control diet for 21 weeks. At this time, three animals were killed by CO2 asphyxiation and their colons

*Abbreviations: CHA-diet, cholic acid diet; ACF, aberrant crypt foci; AOM, azoxymethane.
removed and evaluated for ACF (CO-1 group). The remaining animals were divided into two groups. Five animals were maintained on the control diet (CO-2 group), while six animals were switched to 0.2% CHA-diet (CHA group). After 3 weeks on their respective diets, all animals were killed and their colons removed and evaluated for number, size and multiplicity of ACF.

Animals were killed by CO₂ asphyxiation. Colons were removed, fixed in 4% paraformaldehyde, and prepared for enumeration of ACF as previously described (1-3). The number, size, distribution, and crypt multiplicity of ACF were determined for the entire length of the colon. To determine size, an ocular grid was used to measure the approximate area occupied by the ACF as viewed at 100X magnification and to determine crypt multiplicity, the number of crypts in each focus were recorded.

Analysis of variance (ANOVA) was used in combination with Duncan’s multiple range test to assess the effect of different treatments on the number and growth features of ACF belonging to different groups.

The average body weight of the animals at the time of intervention with the CHA-diet was 597 ± 8.0. After 3 weeks of feeding either the CO diet or CHA-diet the average body weight of the rats were 620 ± 9.0 and 615 ± 8.0 respectively.

The average total number of ACF in the colons of rats that were injected with AOM and then allowed to eat a control diet (CO-1), was approximately 202. Administration of CHA-diet for 3 weeks following 21 weeks of the control diet significantly reduced total number of ACF from 202 to 70. The group that continued to receive the control diet (CO-2), did not differ from the CO-1 group (Table I).

Distribution of the number of ACF with varying crypt multiplicity for the three groups is shown in Table I. The CHA-diet significantly reduced the number of ACF consisting of 1–4 crypts/focus, whereas ACF with higher crypt multiplicity (>5) were not affected (Table I).

Growth features of ACF with 1–4 crypt multiplicity were further scrutinized and divided into different size categories (Table II). Although ACF had similar crypt multiplicity, they varied a great deal in size, i.e. some ACF consisted of large, dilated crypts, whereas others consisted of smaller, more constricted crypts. ACF with 1–2 crypts were more susceptible to elimination by CHA-diet regardless of their size. This trend was not evident for ACF with 3–4 crypts (Table II). A significant reduction was evident only among the ACF that were >5–10×10⁻² mm² in size.

This study demonstrates that feeding a diet containing 0.2% CHA at a late time point significantly reduced the number of ACF in the colons of rats initiated with a colon carcinogen. The CHA intervention significantly reduced those ACF that contained 1–2 crypts per focus, but had less effect on those with a crypt multiplicity of 3–4 or >4. In addition, ACF containing 1–4 crypts that persisted in the colons of animals of ACF, whereas ACF with higher crypt multiplicity ( >5) were not affected (Table I).

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fed CHA tended to be considerably smaller in size than those fed control diets.

Features of ACF, which are quantified by topographic evaluation of colonic mucosa are the number and size of ACF and number of crypts present in each focus (crypt multiplicity). The number of ACF may indicate the number of initiated lesions. A dose response relationship exists between the carcinogen dose and number of ACF induced (2-3). The growth features of ACF can be used to assess the preneoplastic state of the colon. Colonos possessing a higher number of ACF with higher crypt multiplicity appear to be at higher risk for developing colonic tumours than those with a lower number of ACF with higher crypt multiplicity, as long as these lesions are growing at a similar rate. Recent studies have demonstrated that cry paternal multiplicity of ACF predicted the tumour outcome more reliably than the total number of ACF (12-14). The ability of ACF with more advanced growth features to resist the effect of CHA-diet supports the proposal that they represent an advanced preneoplastic state. Recently it has been demonstrated that ACF with advanced growth features also resisted apoptotic cell death induced by AOM (15). These findings suggest that ACF with increasing crypt multiplicity, acquire augmented ability to resist and or adapt to the adverse environmental influences as purported to occur in preneoplastic lesions during liver carcinogenesis (16). The possibility that the ACF with a crypt multiplicity of 1-4, which were not eliminated or remodelled, are biologically more stable lesions and that they may eventually progress into more advanced ACF and tumours, remains to be established.

Size of an ACF quantifies the area occupied by each ACF, which may vary depending on the number of crypts in the focus and the size of each crypt. Previously we reported that CHA treatment not only reduced the number of ACF, but significantly reduced the average size of each ACF (9,10). Interestingly, average crypt multiplicity (mean AC/focus) did not change in groups fed the CHA-diet compared with the control group (9,10) suggesting that ACF with a large area, consisting of more dilated crypts were not present in the group fed the CHA-diet whereas they were present in the group fed the control diet (CO-1 and CO-2). One plausible explanation is that perhaps the ACF with dilated crypts were preferentially eliminated or remodelled by the CHA-diet. This proposal was supported significantly only when ACF with 3-4 crypt multiplicity were considered, whereas in the case of ACF with 1-2 crypt(s), multiplicity reduction was seen in all size categories with highest reduction in the group with the largest size. Recently we demonstrated that a low dose of AOM (5 mg/kg) induced ACF with larger crypts than a high dose of AOM (20 mg/kg) (17). ACF induced by two different doses of AOM respond differently to growth modulation by dietary lipids (17). The complex nature of the disease process prohibits us from speculating further on the significance of this observation. Whether ACF with dilated crypts histologically and developmentally represent more unstable lesions than those with more constricted crypts should be determined in future studies.

The approach of studying the effect of cholic acid on the stability of ACF, 21 weeks after a single injection of AOM provided an unique opportunity to explore the effect of cholic acid on ACF with different growth features. The intention of the study was also to determine if the experimental outcome would be compatible with the notion that ACF are preneoplastic lesions. If ACF are preneoplastic lesions then ACF of different growth features would be expected to represent different preneoplastic state. A corollary exists between the developmental features of ACF and those expected to be present in a population of preneoplastic lesions induced in close proximity to each other. Carcinogenesis is a multistep process involving sequential clonal selection and expansion of initiated cells. During these steps, preneoplastic lesions encounter negative and positive growth control. Those lesions that overcome the negative growth control, enter the next stage and gradually acquire complete growth autonomy. Therefore, as the time progresses, an organ with a large number of initiated lesions is expected to have preneoplastic lesions exhibiting different growth features. In the present investigation, colons of animals injected with a single dose of AOM did have ACF exhibiting different growth features at a later time point and they responded differently to the reducing effect of CHA-diet. ACF with increasing crypt multiplicity were less sensitive to the CHA-diet than those with lower crypt multiplicity. These findings support the notion that ACF with increasing crypt multiplicity represent lesions with increasing preneoplastic potential.

In conclusion, we have demonstrated that a diet containing cholic acid, known to exert a hyper proliferative response in the normal colonic epithelium, eliminates or remodels preferentially ACF with 1-2 crypt multiplicity and a proportion of ACF with 1-4 crypt multiplicity with large dilated crypts. Aberrant crypt foci with increasing crypt multiplicity appear to resist elimination or remodelling by the CHA-diet. The cellular and molecular bases for these observations remain elusive.

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