Intrinsic denervation of the colon is associated with a decrease of some colonic preneoplastic markers in rats treated with a chemical carcinogen

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Denervation of the colon is protective against the colon cancer; however, the mechanisms involved are unknown. We tested the hypothesis that the denervated colonic mucosa could be less responsive to the action of the chemical carcinogen dimethylhydrazine (DMH). Three groups of 32 male Wistar rats were treated as follows: group 1 (G1) had the colon denervated with 0.3 mL 1.5 mM benzyldimethyltetradecylammonium (benzalkonium chloride, BAC); G2 received a single *ip* injection of 125 mg/kg DMH; G3 was treated with BAC + the same dose and route of DMH. A control group (Sham, N = 32) did not receive any treatment. Each group was subdivided into four groups according to the sacrifice time (1, 2, 6, and 12 weeks after DMH). Crypt fission index, ß-catenin accumulated crypts, aberrant crypt foci, and cell proliferation were evaluated and analyzed by ANOVA and the Student *t*-test. G3 animals presented a small number of aberrant crypt foci and low crypt fission index compared to G2 animals after 2 and 12 weeks, respectively. From the second week on, the index of ß-catenin crypt in G3 animals increased slower than in G2 animals. From the 12th week on, G2 animals presented a significant increase in cell proliferation when compared to the other groups. Colonic denervation plays an anticarcinogenic role from early stages of colon cancer development. This finding can be of importance for the study of the role of the enteric nervous system in the carcinogenic process.

Key words: Colon cancer; Dimethylhydrazine; Benzalkonium chloride; Aberrant crypt foci; ß-catenin; Megacolon

Introduction

It has been reported that constipation may be an important factor in the pathogenesis of colorectal cancer because it prolongs the contact of colonic cells with metabolic and dietary carcinogens (1). Constipation is a major symptom of megacolon that may be caused by the intrinsic denervation of the viscera. Available human data have shown that intrinsic colonic denervation is protective against the development of colon tumors, but the reasons for this protection remain unknown (2,3). We have reported that colonic denervation due to chemical ablation of myenteric neurons (4) or due to *Trypanosoma cruzi* infection (5) is associated with a lower incidence of colonic cancer induced by a chemical carcinogen. One challenging hypothesis to explain this phenomenon is that the megacolon mucosa is less responsive to the action of chemical carcinogens and thus less favorable to the development of tumors. In this experiment, we tested this hypothesis by analyzing the effects of the carcinogen 1,2-dimethylhydrazine (DMH) on histological parameters that are believed to precede the development of chemically induced tumors in

rodents, i.e., the formation of aberrant crypt foci (ACF), the crypt fission index (CFi), and the presence of ß-catenin accumulated crypts (BCAC).

Colon cancer can be experimentally induced in rats by the application of DMH. By using this carcinogen, Bird (6) has shown that ACF are precursor lesions of chemically induced colon cancer. At light microscopy the ACF are larger and have a thicker epithelial lining than normal crypts (6). The ACF assays have been used for the detection of factors that influence the modifiers of early colorectal carcinogenesis in rats treated with the chemical carcinogen DMH (7).

The gastrointestinal tract epithelium is characterized by an active and rapid cell turnover, which is closely related to a high proliferative activity in the basal crypt cells (8). One of the first steps in the multistage colonic carcinogenesis is the increased cell proliferation of the colonic crypts. The crypts themselves also reproduce by a process called crypt fission, beginning with a basal bifurcation and followed by a longitudinal division (9). It is thought that an increased rate of crypt fission, rather than hyperproliferation, is critical in precancerous gastrointestinal states (10).

It has been shown that ß-catenin protein accumulation is involved in the small dysplastic crypts observed on histological sections of the rat cancer-predisposed colon, and such crypts with excessive ß-catenin have been designated as BCAC (11). BCAC might be premalignant lesions in the colon, and seem to be more likely to progress to a malignant transformation when compared with ACF (12).

In view of these considerations, we studied the development of ACF, epithelial cell proliferation, CFi, and expression of ß-catenin after a single carcinogenic dose of DMH administered to denervated and non-denervated rats.

Material and Methods

Animals

Ninety-six male Wistar rats supplied by the Ribeirão Preto School of Medicine, weighing 250 g, were housed 1 or 2 per cage in a temperature-controlled room at 24 ± 1°C and maintained on a 12:12-h light-dark cycle. All animals had free access to the same diet, a commercial chow and tap water. The animals were maintained in agreement with the guidelines of the Committee on Care and Uses of Laboratory Animals of the National Research Council of the NIH (USA).

Experimental design

The animals were divided into three groups of 32 animals each: group 1 (G1) was treated with benzyldimethyltetradecylammonium (benzalkonium chloride, BAC),

group 2 (G2) was treated with DMH, and group 3 (G3) was treated with BAC + DMH. The control group (Sham, N = 32) did not receive any treatment. Each group was then subdivided into four groups according to the time of sacrifice (1, 2, 6, and 12 weeks after receiving the carcinogen injection). Animals from G1 and G3 were anesthetized with ketalar (86.5 mg/kg body weight) and rompum (10 mg/kg body weight) and laparotomized. In the animals from G1 and G3, the distal colon was taken out of the peritoneal cavity and wrapped with cotton soaked in 0.3 mL of a 1.5-mM BAC solution (Sigma, St. Louis, MO, USA) in saline for 30 min, or with saline alone in the control groups. After treatment, the intestinal segment and the entire peritoneal cavity were rinsed with 0.9% saline at 36°C, and the abdominal cavity was closed, as previously described (13). The animals from G2 and G3 received a single *ip* injection of 125 mg DMH (Wako Pure Chemical Industries, Osaka, Japan) per g body weight (6). The animals from G3 received the DMH injection 10 days after BAC treatment.

Morphological analysis

The animals were killed by carbon dioxide asphyxiation. During autopsy an 8-cm segment was removed from the distal colon of each rat, opened longitudinally, and fixed flat on a styrofoam board in 10% neutral buffered formalin for 24 h and then stained with 0.2% methylene blue (6). The numbers of ACF and crypt fissions were counted per microscopic field at low power magnification by light microscopy. The analysis was conducted in a blind fashion regarding the groups.

Immunohistochemical analysis

The sections were heated at 60°C for 75 min, deparaffinized twice in xylene for 7 min, and rehydrated through graded alcohols. Antigen retrieval was carried out by heating the sections in 10 mM citrate buffer, pH 6.6, in a pressure cooker, as previously described (13). The slides stored at 4°C were held at full pressure in a pressure cooker for 3 min, and the slides kept at room temperature were held at full pressure for 10-15 min to obtain optimal results (membranous expression of ß-catenin in the normal adjacent colonic mucosa and nuclear expression of proliferative cellular nuclear antigen (PCNA)). To prevent nonspecific staining, the sections were incubated in a blocking solution of 10% normal horse serum in PBS (10 mM sodium phosphate, pH 7.4, and 0.137 M NaCl) for 15 min. The sections were incubated for 1 h at 37°C in a humidified chamber with mouse monoclonal anti-ß-catenin antibody (IgG1; Transduction Laboratories, Lexington, KY, USA) diluted 1:2000 in a blocking solution. To estimate colonic cell proliferation, thin slices (4 mm) from the paraffin-embedded colon of all animals were

immunostained with anticyclin/PCNA antibody (PCNA/No-vocastra, Newcaste-upon-Tyne, UK), diluted 1:2000 in a blocking solution (5).

The control sections were incubated with mouse mono-clonal antibromodeoxyuridine (IgG1; Chemicon, Temecula, CA, USA) or with normal horse serum at the same concen-tration as the primary antibody for a negative control in every set of stained slides. The remaining procedures took place at room temperature. The sections were washed in PBS, incubated for 30 min with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in a blocking solution, and treated with 3% hydrogen peroxide in 30% methanol for 10 min to stop endogenous peroxidase activity. After washing in distilled water, the sections were incubated for 30 min with a streptavidin-biotinylated horseradish peroxidase complex (Amersham Corp., Arlington Heights, IL, USA) diluted 1:100 in a block-ing solution, washed in PBS, and incubated with the sub-strate, 3'-diaminobenzidine (Sigma). The slides were coun-terstained with 0.1% methyl green for 3 min, dried, and mounted with 50% Clearium/50% xylene (Surgipath Medi-cal Industries, Inc., Richmond, IL, USA).

Cell nuclei were counted in 100 colonic crypts of each colon. The PCNA-labeling index (PCNA-Li) is reported as the ratio of positively stained nuclei to the total number of nuclei counted per 100 crypts.

Statistical analysis

Data are reported as means ± SEM. Statistical analysis was performed by ANOVA and the Student t-test, with the level of significance set at $P < 0.05$.

Results

All animals remained in good health and none showed clinical signs of nutritional deficiency during the experi-mental period. At the end of the experiment the average weight of the animals did not differ between the two groups.

In the macroscopic analysis all the animals submitted to the application of BAC presented a discrete enlarge-ment of the colon. No tumors or polyps were observed on the colonic mucosa of the rats.

The histopathological study showed that the animals submitted to BAC application presented a decrease in the number of myenteric neurons when compared to the ani-mals submitted to saline.

The effect of time on the number of ACF in the distal colon of the rats is presented in Figure 1. ACF were only observed in G2 and G3 animals, with their numbers in-creasing between 1 and 12 weeks after treatment with the carcinogen. In the 1st week the number of ACF was similar

in both groups but after the 2nd week G3 presented a smaller number of ACF compared to G2.

The colons of both groups of rats examined at 1 and 2 weeks post-treatment contained ACF consisting mainly of 1 or 2 crypts. The percentage of ACF consisting of 4 or more crypts was significantly greater at 6 and 12 weeks after treatment with the carcinogen, but ACF growth was slower in G3 than in G2, as shown in Figure 2.

CFi was significantly greater in G1 and G3 in the first 2 weeks compared to G2. However, in the 6th week G1 and G3 animals presented a reduction in CFi whereas G2
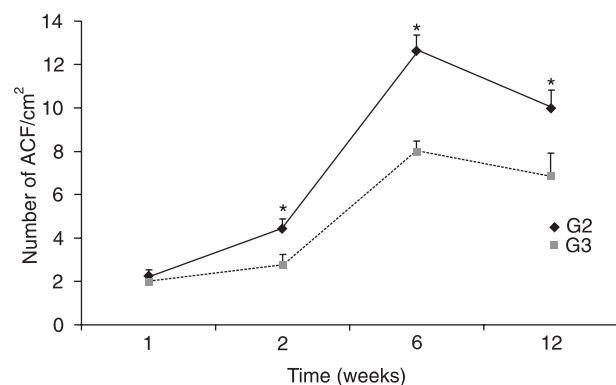

Figure 1. Effect of time on the number of aberrant crypt foci (ACF) in the rat distal colon. G1 = animals submitted to colon denervation with BAC; G2 = animals treated with DMH; G3 = animals with a denervated colon and treated with DMH. BAC = benzyldimethyltetradecylammonium; DMH = dimethylhydrazine. *P < 0.05 compared to G3 (ANOVA).
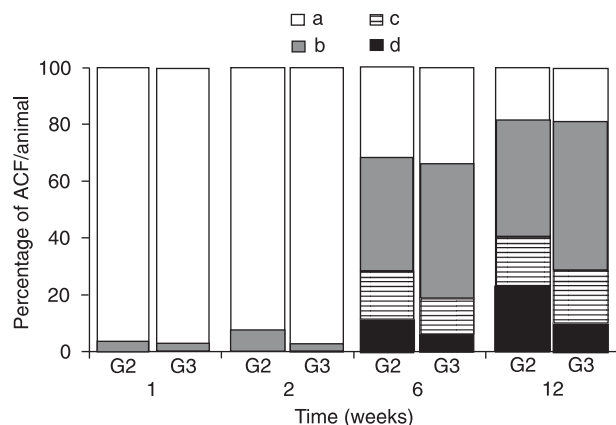

Figure 2. Effect of time on the number of crypts per aberrant crypt foci (ACF) in the rat distal colon. ACF with one (a), two (b), three (c), and four (d) crypts. G1 = animals submitted to colon denervation with BAC; G2 = animals treated with DMH; G3 = animals with a denervated colon and treated with DMH. BAC = benzyldimethyltetradecylammonium; DMH = dimethylhydrazine. The number of ACF with four crypts was significantly higher in G2 than in G3 animals at the 6th and 12th weeks (P < 0.01, ANOVA).

animals presented a remarkable increase in this index, so that at this time point all groups showed a similar CFi. Moreover, twelve weeks after DMH injection the G2 animals showed a higher CFi than the other experimental groups, which were similar to one another (Figure 3).

BCAC were detected in the colons of all groups. The analysis showed morphological alterations such as nuclear atypia and structural abnormality, with ß-catenin immunoreactivity in the cytoplasm and/or nucleus. There
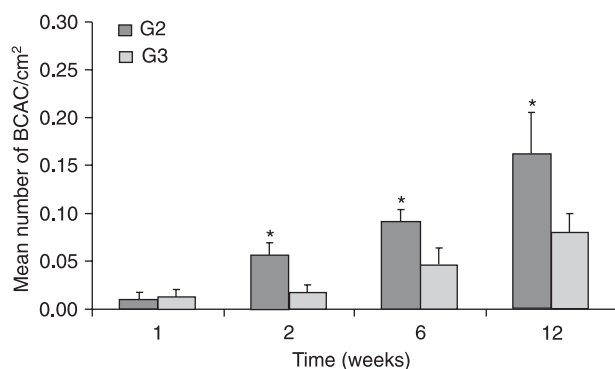
was an increase in the index of ß-catenin crypts (iBCC) of G2 and G3 between 1 and 12 weeks. In the 1st week the iBCC was analogous in both groups but from the 2nd week on it increased more slowly in G3 than in G2, as shown in Figure 4.

The results of PCNA-Li are shown in Figure 5. From the 12th week on G2 presented a significant increase in cell proliferation when compared to the other groups, which were similar to one another. All experimental groups presented a higher PCNA-Li when compared to the sham (normal) animals from the 6th week on.

Discussion

We demonstrated here that denervation of the rat colon was associated with a decreased number of ACF, CFi, number of BCAC, and epithelial cell proliferation after a single injection of a carcinogenic dose of DMH. A large body of evidence indicates that these parameters may be considered to be reliable premalignant lesions (6,9,14). Thus, our results support to the proposed assumption that colorectal carcinogenesis is at least partially prevented by colonic denervation. Furthermore, because the parameters studied here were shown to be altered early during colonic carcinogenesis, we may suggest that colonic denervation is effective during the early stages of colonic

Figure 3. Effect of time on the ß-catenin-accumulated crypts (BCAC) in the rat distal colon. G1 = animals submitted to colon denervation with BAC; G2 = animals treated with DMH; G3 = animals with a denervated colon and treated with DMH. BAC = benzyldimethyltetradecylammonium; DMH = dimethylhydrazine (P < 0.01, ANOVA).
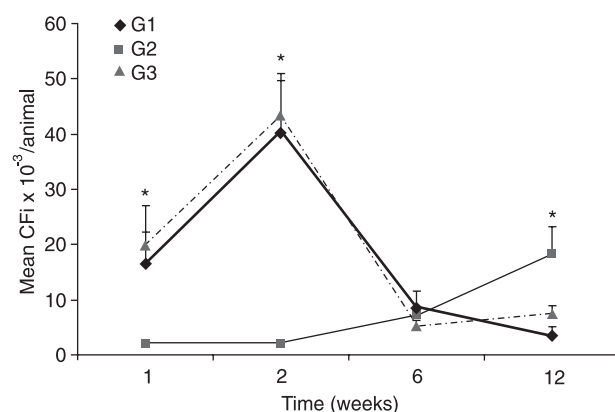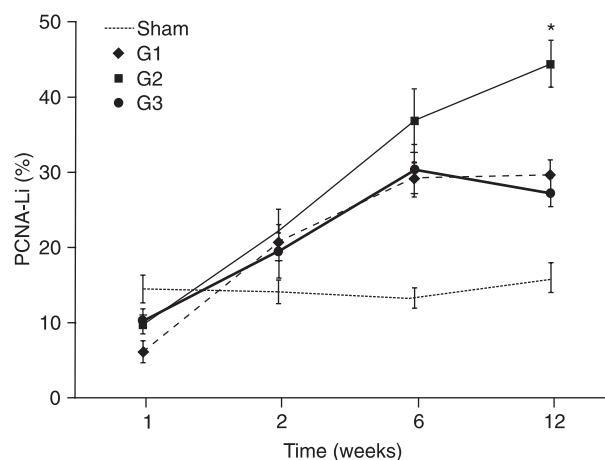

Figure 4. Effect of time on crypt fission index (CFi) in the rat colonic mucosa. G1 = animals submitted to colon denervation with BAC; G2 = animals treated with DMH; G3 = animals with a denervated colon and treated with DMH. BAC = benzyldimethyltetradecylammonium; DMH = dimethylhydrazine. The CFi was significantly smaller in G2 than in G1 and G3 animals at the first and second weeks, and higher at the 12th week (P < 0.01, ANOVA).

Figure 5. Effect of time on the index of proliferating cell nuclear antigen (PCNA) in the distal colons of rats. G1 = animals submitted to colon denervation with BAC; G2 = animals treated with DMH; G3 = animals with a denervated colon and treated with DMH. Sham animals did not receive any treatment. BAC = benzyldimethyltetradecylammonium; DMH = dimethylhydrazine. At 12 weeks, the PCNA-labeling index (PCNA-Li) was higher than in the other experimental groups (*P = 0.03; Student t-test).

cancer development. Moreover, all the parameters studied may be related to each other by not completely known mechanisms, since it is thought that ß-catenin accumulation may be related to the dysregulation of crypt fission (10,15) and that altered crypt fission is a crucial event during ACF formation (16) and all these parameters are dependent on epithelial cell proliferation.

The mean number of ACF was lower in G3 than in G2 after 2 weeks. The number of ACF consisting of 4 or more crypts was significantly higher mainly in G2 at 6 and 12 weeks. This suggests that the number of crypts per ACF increases with time in a large proportion of ACF and is consistent with the hypothesis that ACF undergo crypt multiplication and/or crypt branching (17). The denervated animals presented a slower increase in the number of crypts per ACF.

The CFi was also smaller in G3 animals at 6 and 12 weeks after exposure to the carcinogen. The denervated animals presented a higher CFi at 2 weeks, probably as a consequence of the development of megacolon, but the CFi decreased after 6 weeks, being lower in G2 but increasing over subsequent weeks. After 12 weeks the CFi of G2 was higher compared to the other groups, which were similar to one another.

It is possible that the smaller CFi may be related to the also smaller iBCC in G3 animals when compared to G2, since crypt fission has been proposed to explain the spread of mutated clones in the colonic mucosa (9).

Alterations in the APC or ß-catenin gene are considered to play a gatekeeper role in the development of colon cancers in both humans and rodents (17,18). In the present study, we have demonstrated an association between preneoplastic lesions and BCAC in a rat model of colon carcinogenesis, in agreement with the literature (19,20).

In the stage of carcinogenesis promotion, represented by the period after the 2nd week of treatment, all biomarkers were shown to be higher in the non-denervated than in the denervated animals. Based on the current knowledge of the pathogenesis of colon cancer, we cannot make definitive statements about the mechanisms and factors that might be involved in megacolon for protecting against cancer development. We had previously discussed that, at least in some cases, despite the fact that tumors probably arise from a single cell and are clonal populations, there has to be some sort of cooperativity between the cells for the neoplastic process to begin (21,22). In some cases the mutated clones, once established, spread through the tissues before becoming overtly invasive. There may be instances where such clones expand and remain cohesive, often involving a large tissue area, a phenomenon also called field cancerization (9). The present findings lead us to hypothesize that the action of neuropeptides could leave the denervated colonic mucosa less responsive to the action of chemical carcinogens and thus less favorable to the development of tumors.

A possible hypothesis regarding our experimental model is that the complex interactions among the regulatory peptides could be related to a high resistance of the megacolon mucosa to the action of carcinogens. Peptide containing nerve fibers may be detected in the normal rat colonic mucosa (23) and these peptides play an important role in the control of cell proliferation in normal intestinal mucosal cells (24) and are involved in cancer development (25,26). The denervation of the gut leads to profound changes in the mucosal peptide concentration (23). Thus, it is quite plausible that the combination of neuropeptide concentration with other factors may be able to inhibit the carcinogenic process, since it is well known that the gut neuroendocrine system is significantly altered in the denervated colon and in megacolon (27-30).

In addition to playing a role in cell proliferation (31,32), neuropeptides and other regulatory peptides may have a possible effect on the control of adhesion between neighboring epithelial cells (33) and on catenin regulation, which is a crucial process related to colonic carcinogenesis (34). Interestingly, it has been observed that adherens junctions and tight junctions are regulated by gastrin in epithelial cells, a fact that may contribute to the co-carcinogenic role of this prohormone in colorectal carcinoma (35). We have previously observed that gastrin level is influenced by gastric denervation (36), but it remains to be elucidated if it is also affected by colon denervation.

In view of these considerations, we conclude that studying the carcinogenic process in the intrinsically denervated colon can be of importance not only in clarifying new aspects regarding colonic cancer pathogenesis, but also in providing an experimental model for assessing the role of the nervous system in the carcinogenic process, and hopefully leading to the investigation of new preventive strategies.

Acknowledgments

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