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Promotion of intestinal carcinogenesis by dietary methionine

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The metabolism of the polyamines spermidine and spermine is known to be enhanced in rapidly proliferating cells. Methionine is a precursor of the aminopropyl moieties of these amines. Therefore, it was of interest to study the effects of a methionine supplemented diet on polyamine metabolism and preneoplastic changes occurring in the intestinal tract of rats treated with the chemical carcinogen azoxymethane (AOM). Adult Wistar rats received 15 mg AOM/kg body wt (i.p.) once each week for 2 weeks. Thereafter, the rats were randomly divided into two groups and received controlled isoenergetic diets containing the same amount of folate, choline and vitamin B_{12} during 12 weeks: one group was kept on a standard diet; the other was fed the same diet, except that 1% L-methionine was added at the expense of carbohydrates. After 12 weeks, the administration of the methionine-supplemented diet stimulated the turnover rate of ileal epithelial cells, indicating enhanced crypt cell proliferation. Furthermore, in this group, a 2-fold increase in the number of aberrant hyperproliferative crypts and the appearance of tumors was observed in the colon. These effects were accompanied by the increased formation of spermidine and spermine due to the enhancement of *S*-adenosylmethionine decarboxylase activity and by the upregulation of Cdx-1, a homeobox gene with oncogenic potentials. The experimental data do not support the view of a chemopreventive effect of dietary methionine supplementation on intestinal carcinogenesis in rats, even at an early phase of preneoplastic development, but rather suggest that methionine promotes intestinal carcinogenesis.

Introduction

Dietary factors are known to be among the most important environmental risk factors for cancer while food components are also regarded as one of the main chemopreventive agents in the prevention of cancer (1). In a recent US study increased risk of colorectal cancer was associated with a diet low in folate and methionine (2). In developing countries, epidemiological studies linked diets low in methionine, choline and folate to primary hepatoma (3). In rodents prolonged intake of methionine-deficient diets, even without exposure to any known carcinogen, has been shown to result in the development of liver tumors (4), whereas diets supplemented with choline and methionine seemed to prevent or at least diminish the effects of some chemical carcinogens (5) and prolong the survival of spontaneously leukemic AKR mice (6). In contrast, cancer cell lines of different origins exhibited a strict dependency on methionine for growth, suggesting an important relationship to oncogenic development (6–8). However, after appearance of primary tumors in rats, rhabdomyosarcoma metastatic spread to the lungs was inhibited by a low methionine diet (9). Neither the biochemical basis of methionine dependence of established tumors and cancer cells, nor the potential chemopreventive effects of methionine are understood. Methionine plays a critical role in cell development because it is the precursor of *S*-adenosylmethionine which is the primary methyl-group donor in a great variety of methylation reactions (10) and the precursor of the aminopropyl moieties of spermidine and spermine (11). These amines play a central role in cellular growth and differentiation (12). Polyamines are involved in many steps of DNA, RNA and protein synthesis. Tumor cells exhibit a very high requirement for these molecules in order to sustain cell growth through elevated *de novo* synthesis and enhanced uptake from the extracellular environment (13). In this regard, dietary polyamines have a direct modulatory effect on preneoplastic promotion in the intestinal mucosa (14,15). *S*-adenosylmethionine decarboxylase (AdoMetDC) and ornithine decarboxylase (ODC) are key enzymes of polyamine biosynthesis (11). In view of the significant involvement of polyamines in intestinal carcinogenesis and the apparent importance of methionine as a tumor growth regulator, the aim of this study was to determine the effects of a methionine supplemented diet on preneoplastic histopathological changes in the intestinal tract of rats treated with the carcinogen azoxymethane (AOM) and on changes in intestinal polyamine metabolism. Our data show that feeding increased amounts of methionine strongly promoted intestinal carcinogenesis and favored polyamine synthesis. In addition, the methionine-supplemented diet caused the upregulation of Cdx-1, a homeobox gene with oncogenic potentials that is mainly expressed in the intestinal crypts (16,17). Instead of supporting the view that dietary methionine may serve as a chemoprotectant against carcinogens (6), or that a diet that increases the availability of methyl-groups reduces the incidence of colorectal cancer (2,18), the present results suggest that intestinal carcinogenesis is promoted by a methionine-rich diet.

Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; AOM, azoxymethane; DAO, diamine oxidase; ODC, ornithine decarboxylase.

Materials and methods

Animals and diets

The experiments were conducted according to the National Research Council Guide for Use and Care of Laboratory Animals with the authorization (no. 00573) of the French Ministry of Agriculture.

Male Wistar rats (n = 30) weighing 230–245 g were housed under standardized conditions (22°C; 60% relative humidity; 12 h light/dark cycle, 20 air changes/h) and fed a standard diet with free access to drinking water.

All animals received i.p. injection of 15 mg AOM/kg body wt once each week for 2 weeks. The rats were randomly divided into two groups which received controlled isoenergetic diets (234 kcal/kg/day) during 12 weeks.

The control group ($n = 15$) was kept on the standard diet. The standard diet contained 13.5% protein as casein and fish protein, 62% carbohydrates as wheat starch, 3% lipids as soya and fish oil, 6% salt mixture and 1% vitamin mixture (UAR A05, Villemoisson/Orge, France). The total methionine content of this diet was 0.2%. The animals of the other group ($n = 15$) were fed the same diet containing the same amount of choline (0.2%), folate (0.05%) and vitamin B_{12} (1 μg/g), except that 1 g of L-methionine (Sigma-Aldrich, Saint Quentin Fallavier, France) was added per 100 g of diet at the expense of wheat starch. After 12 weeks of controlled feeding, the body weights of the animals in the two groups showed no significant changes and were, respectively, 524 ± 8 g in the controls versus 504 ± 13 g in the methionine-supplemented group. The entire colon and a 10 cm segment corresponding to the terminal part of the ileum were collected under anaesthesia, 12 weeks after initiation of controlled feeding, for histological and biochemical analyses.

Assessment of aberrant crypts and tumors in the colon

The determination of aberrant hyperproliferative crypts and tumors were performed on a segment 5 cm in length corresponding to the distal part of the colon. The segment was washed with physiological saline, cut open, pinned out flat and fixed in 10% buffered formalin. The colon was stained with 0.2% methylene blue for 5 min, rinsed in Krebs-Ringer buffer, placed onto a glass slide and examined microscopically using a low power objective (×40) for assessment of the number of aberrant crypts and of the presence of tumors (19,20).

The criteria for the identification of aberrant crypts were: (i) an increased size; (ii) a thicker epithelial cell lining; and (iii) an increased pericryptal zone relative to normal crypts.

Enzymes of polyamine metabolism

Colonic mucosal samples were homogenized in 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, 0.5 μM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 33 000 *g* for 25 min at 4°C, the supernatants were collected and ODC, AdoMetDC and diamine oxidase (DAO) assays were performed rapidly. ODC activity was evaluated by measuring the rate of $^{14}CO_2$ formation from L-[1-^{14}C]ornithine (55 mCi/mmol; Amersham, Les Ulis, France) (21) and AdoMetDC activity was determined by measuring the rate of $^{14}CO_2$ formed from *S*-adenosyl L-[carboxyl-^{14}C]methionine (60 mCi/mmol; Amersham) (22). DAO determination was based on the formation of radioactive toluene-extractable oxidation products of [1, 4-^{14}C]putrescine (118 mCi/mmol; Amersham) (23).

Determination of the polyamines

Colonic mucosal samples were homogenized in 10 parts (w/v) 0.2 M perchloric acid and the homogenates were centrifuged at 3000 *g* for 10 min after standing for 16 h at 2°C. The clear supernatants were diluted with 0.2 M perchloric acid and 200 μl aliquots were applied on a reversed-phase column for separation. The polyamines (putrescine, spermidine and spermine) were determined by separation of their ion pairs formed with *n*-octanesulfonic acid, reaction of the column effluent with *o*-phtalaldehyde/2-mercaptoethanol reagent and monitoring of fluorescence intensity (24).

Nuclear DNA labelling

Epithelial cell migration rate from crypt base to villus tip was measured in five animals from each group. The rats were injected i.p. with [^{3}H]thymidine (300 μCi/kg body wt, 81 Ci/mmol; Amersham) 17 h before being killed. Labeling of nuclear DNA was revealed *in situ* in ileal samples. Tissue sections (5 μm) embedded in paraffin were coated with the photographic emulsion EM-1 (Amersham) for high resolution microautoradiography and exposed for 4 weeks in the dark. The villus-crypt height and the position of the silver grains related to the crypt base were determined with an image analyzer (Biocom, Les Ulis, France).

RNA analysis by RT–PCR

Mucosal samples of the colon were scraped off with glass slides and RNA was extracted as previously described (25). RT–PCR was performed as previously described in detail (26,27) using the following primers to detect the Cdx-1, Cdx-2 and β-actin transcripts: CDX1a(dGTAAGACTCGGACCAAGGACAAGTA), CDX1b(dAACTGTGTGGGAGGCATGGGCTGCG), CDX2a(dCCCAGCGGCCAGCGGCGAAACCTGT), CDX2b(dTATTTGTCTTTTGTCCTGGTTTTC), ACTa(dATATCGCTGCGCTCGTCGTCGACAA) and ACTb(dAACACAGCCTGGATGGCTACGTACAT). The synthetic oligonucleotides were from Gibco BRL (Cergy Pontoise, France). Amplification was performed for 20–40 cycles to overlap the range of cycles in which the amount of PCR products increased exponentially. PCR products were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide and analyzed using an imaging densitometer (Bio-Rad, Ivry sur Seine, France). The RT–PCR products were cloned into the pGEMT plasmid (Promega, Charbonnières, France) and sequenced to confirm their identity.

Table I. Number of aberrant crypt foci (ACF) and tumors (T) in the distal colon (5 cm length) of AOM treated rats fed for 12 weeks with a standard diet or with the methionine supplemented diet

Groups	ACF		T	
	Incidence[a]	ACF[b]	Incidence[a]	T[c]
Standard diet	(10/10)	20 ± 1.4 (16–23)	(0/10)	0
Diet + 1% methionine	(10/10)	35 ± 2.5[d] (21–43)	(5/10)	2.5 ± 0.5 (1–4)

Values are means ± SE. Range of values appears in parentheses. The diet composition is given in Materials and methods.
[a]Number of rat colon with aberrant crypt foci or tumors divided by total number of colons scored.
[b]Number of aberrant crypt foci per cm length.
[c]Number of tumors (adenomas) per colon.
[d]$P < 0.01$ (Student's *t*-test).

Table II. Effect of dietary methionine on epithelial cell migration in the ileum of rats treated with a chemical carcinogen

Dietary groups	H[a] (μm)	h[b] (μm)	H/h (%)
Standard diet	502 ± 12	189 ± 3	36
Diet + 1% methionine	491 ± 8	211 ± 3[c]	45

The rats were injected i.p. with [^{3}H]thymidine (300 μCi/kg body wt) 17 h before being killed. The villus–crypt height and the position of the silver grains related to the crypt base were determined with an image analyzer. Values are means ± SE for 30 villus-crypt units/animal counted in five animals/dietary group.
[a]Total crypt-villus height.
[b]Distance of the front of the labeled cells from the crypt base.
[c]$P < 0.05$ (Student's *t*-test).

Statistics

Data are reported as means ± SE. Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using Student's *t*-test.

Results

Effect of methionine supplementation on the formation of aberrant crypt foci and tumors

As shown in Table I, all rats injected with AOM developed numerous abnormal and hyperplasic colonic crypts, regardless of the dietary treatment. However, the administration of the diet supplemented with 1% methionine resulted in a 2-fold increase in the number of aberrant colonic crypts, when compared with animals fed the standard diet. After 12 weeks of feeding the methionine-supplemented diet, adenomas appeared in the colon of 50% of the rats, whereas in animals fed with the standard diet the colon remained tumor free.

Effect of diet on intestinal epithelial cell migration

Since AOM promotes hyperproliferative changes in the colon but also in the small-intestine, we examined the migration rate of the ileal epithelial cells along the crypt–villus axis as a marker of epithelial cell turnover. Rats were treated with AOM for 2 weeks and then fed for 12 weeks with the standard diet or with the same diet supplemented with methionine and injected i.p. with [^{3}H]thymidine 17 h before being killed (Table II). Autoradiography analyses performed on histological

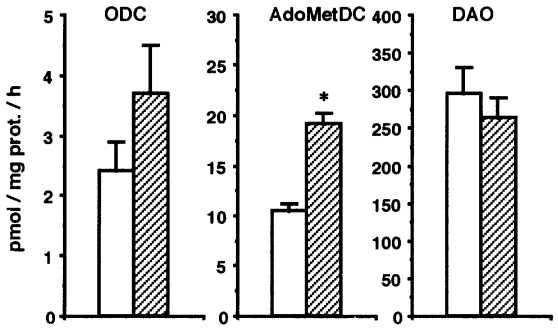

Fig. 1. Activity of ODC, AdoMetDC and DAO in the colonic mucosa of rats treated with the chemical carcinogen (AOM) and then fed for 12 weeks with either the standard diet (open column) or with the same basic diet supplemented with 1% L-methionine (hatched column). Values are given in pmol/mg protein/h (means ± SE, $n = 10$ per group). *$P < 0.01$.

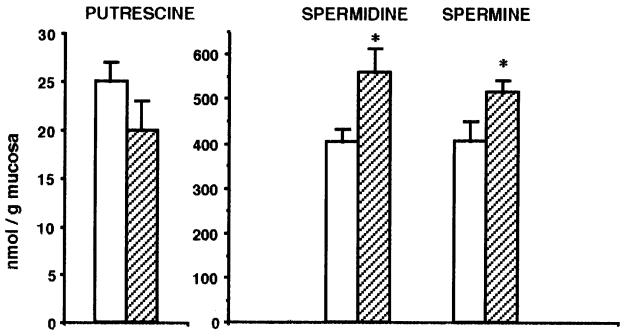

Fig. 2. Polyamine content of the colon mucosa of rats treated with the chemical carcinogen (AOM) and then fed for 12 weeks with either the standard diet (open column) or with the same basic diet supplemented with 1% L-methionine (hatched column). Values are given in nmol/g mucosa (means ± SE, $n = 10$ per group). *$P < 0.05$.

sections showed that the front of labeled epithelial cells were located up to 45% of the crypt–villus axis in the ileal mucosa of rats fed with the methionine-supplemented diet. In animals fed with the standard diet, the front of labeled cells reached only 36% of the crypt–villus height. These data indicate that feeding the methionine-supplemented diet enhances the migration rate of the intestinal epithelial cells along the crypt–villus axis as a result of enhanced cell turnover in the proliferative compartment.

Activity of enzymes of polyamine metabolism

The activities of the two rate-limiting enzymes of polyamine synthesis, ODC and AdoMetDC, and the activity of DAO, the enzyme involved in polyamine and putrescine catabolism, were measured in the colonic mucosa (Figure 1). In rats receiving the methionine-supplemented diet a 2-fold increase of AdoMetDC activity was observed. ODC activity was not significantly increased when compared with animals fed with the standard diet. The activity of DAO remained unaffected by the treatment. These results show that methionine supplementation favors polyamine biosynthesis through the activation of AdoMetDC.

Polyamine content in the mucosa of the colon

The rats treated with AOM and then fed for 12 weeks with the methionine supplemented diet showed a significant increase in the mucosal content of spermidine and spermine as compared with controls (Figure 2). The amounts of spermidine and spermine were enhanced by 40 and 30%, respectively. The mucosal content in putrescine was not significantly modified by the dietary treatment.

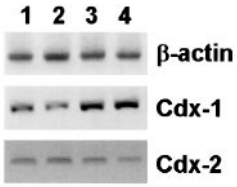

Fig. 3. Representative Cdx-1 and Cdx-2 mRNA patterns in the colon mucosa of rats treated with the chemical carcinogen (AOM) and then fed for 12 weeks with either the standard diet (lanes 1 and 2) or with the same basic diet supplemented with 1% L-methionine (lanes 3 and 4). RT–PCR was performed for 22 cycles to detect the β-actin mRNA and for 36 cycles to detect the Cdx-1 and Cdx-2 transcripts.

mRNA expression of the Cdx-1 and Cdx-2 genes

The intestine-specific Cdx-1 and Cdx-2 genes participate in the control of epithelial cell proliferation and differentiation. Semi-quantitative RT–PCR analyses of the Cdx-1 and Cdx-2 mRNAs were performed on samples prepared from the colonic mucosa of controls and rats fed the methionine-rich diet and were related to the amount of β-actin mRNA. A clear upregulation of the Cdx-1 mRNA was observed in rats fed with the methionine-supplemented diet compared with control animals (Figure 3). However, no significant changes were seen in the level of Cdx-2 mRNA.

Discussion

The chemical carcinogen AOM induces colonic adenomas and adenocarcinomas and also to a lesser extent small intestinal tumors (28). Administration of AOM caused a continuum of morphological changes from normal intestinal epithelium to carcinoma, which are biologically and histologically quite similar to those seen in humans (28). Because of the potential progression of early changes to malignancy, the study of the premalignant hyperproliferative lesions and aberrant crypts is crucial for the understanding of the pathogenesis of colon cancer. In this regard, the identification of dietary factors that are able to modulate such a premalignant process might have important consequences on the management of anticancer therapy. Among these factors, methionine might represent an interesting tool in the control of neoplastic process. It was suggested in several reports (2,5,6,18) that methionine and/or methionine-related metabolites (choline, folates) may reduce the incidence of colorectal cancer and that ‘feeding of large amounts of choline and/or methionine may possibly change oncogenic cells back to normal’ (5). However, our present results do not support this hypothesis, since we present evidence that a diet supplemented with methionine is hastening the appearance of intestinal preneoplastic changes and tumorigenesis. Indeed, in the ileum of rats fed the methionine-rich diet, the turnover rate of crypt cells was stimulated, as shown by the increased migration rate of the epithelial cells along the crypt–villus axis. In the colonic mucosa of these rats an increased number of hyperproliferative aberrant crypts and formation of adenomas were observed.

It has been proposed that the amount of methionine in the diet, through its effects on the formation of *S*-adenosylmethionine, might directly affect the methylation pattern of DNA, with consequent changes in the expression of genes that play critical roles in the regulation of growth and differentiation and which are involved in the modulation of carcinogenesis

(29). Alternatively, we show in the present report that the preneoplastic intestine uses extensively methionine in order to meet the high requirement of endogenous polyamines for the carcinogenic process (13–15). Indeed, dietary methionine supplementation increased AdoMetDC activity in the intestinal mucosa indicating that methionine supplementation triggered the stimulation of the polyamine biosynthesis by increasing the availability in decarboxylated *S*-adenosylmethionine which through its aminopropyl part directs the synthesis of spermidine and spermine (11). This is attested by the enhanced amount of spermidine and spermine measured in the colonic mucosa of animals receiving the methionine-supplemented diet. In view of the known enhanced requirement of polyamines for the support of sustained growth of tumor cells and other rapidly proliferating cells (12–15), the methionine-triggered enhancement of polyamine synthesis may also be crucial in the processes involved in carcinogenesis. Our results suggest that, as for established tumors (8,9), cells at a very early stage of the neoplastic process might be dependent on polyamine availability and that dietary methionine might be used by the cells in order to favor the polyamine biosynthetic pathway and consequently cell proliferation.

The Cdx-1 and Cdx-2 homeobox genes play critical roles in the control of intestinal cell proliferation and differentiation (26,27,30) and their involvement in colon cancers has recently been proposed. Indeed, Cdx-1 has oncogenic potentials (16). Its inhibition in human colonic cancer Caco-2 cells reduces cell proliferation (27) and ectopic Cdx-1 expression accompanies intestinal metaplasia in the oesophagus and stomach (17). Inversely, Cdx-2 seems to be a tumor-suppressor gene (31). Although no target of Cdx-1 regulation has been identified so far in the intestine, it should be emphasized that Cdx-1 upregulation is often associated with elevated cell proliferation such as the precocious intestinal maturation in suckling rats due to polyamine administration (J.-N.Freund and Peulen, unpublished data) and with intestinal morphogenesis and crypt hyperproliferation triggered by retinoic acid (32). Therefore, we propose that the higher expression of Cdx-1 in rats fed the methionine-supplemented diet is linked with the increased cell turnover and that it may subsequently potentiate the tumorigenic effect of the chemical carcinogen. As far as Cdx-2 is considered, we have not found any significant modification of its pattern in the bulk colonic mucosa of animals exhibiting preneoplastic development. However, its involvement in carcinogenesis is supported by the fact that Cdx-2 repression occurs in human colorectal cancer cells and in high grade adenoma and carcinoma after treatment of rats with a chemical carcinogen (38).

In conclusion, our data do not support the view of a potential chemopreventive effect of enhanced dietary methionine. On the contrary, under our experimental conditions, the increase of methionine supply enhanced polyamine biosynthesis, promoted intestinal carcinogenesis even at an early phase of preneoplastic development and these effects were associated with the up-regulation of the Cdx-1 homeobox gene. Although our observations are suggestive, it is presently not possible to extrapolate directly to humans since differences in the diet composition between the two species may influence the effects of dietary methionine.

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