Dietary Calcium and Vitamin D Modulate 1,2-Dimethylhydrazine-induced Colonic Carcinogenesis in the Rat

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ABSTRACT

To determine whether supplemental dietary calcium and/or vitamin D deficiency are involved in modulating colon cancer induced by 1,2-dimethylhydrazine (DMH), Sprague-Dawley rats were fed diets containing either: (a) a normal content of calcium (0.87%) and phosphorus (0.60%) with 2.2 IU of vitamin D3 per g of feed (group A); (b) the same diet as group A, but with calcium and phosphorus increased to 1.80 and 0.80%, respectively (group B); or (c) a vitamin D-deficient diet with supplemental calcium (1.80%) and phosphorus (0.80%) (group C). After 6 weeks on their respective diets, one-half the animals in each group were given s.c. injections of either vehicle or DMH (20 mg/kg body weight/week) for 26 weeks. Animals were then sacrificed and the incidence of tumors as well as the number of tumors per tumor-bearing rat were determined. Colonic mucosal polyamine levels were measured after 15 weeks of exposure to vehicle or DMH, before development of histologically recognizable neoplasms.

The results of these experiments demonstrated that neither calcium supplementation alone nor supplemental calcium in conjunction with vitamin D deficiency altered the incidence of colonic cancer induced by this carcinogen. Supplemental calcium, however, significantly decreased the number of rats with multiple tumors and reduced tumor size. Moreover, vitamin D deficiency abolished these protective effects of calcium on colon cancer in this experimental model. DMH treatment increased polyamine levels in the premalignant colonic mucosa in group A rats. This carcinogen-induced effect was blunted by high dietary calcium. Vitamin D-deficient, calcium-supplemented rats (group C) showed an increase in N4-acetylspersimidine, but not the other polyamines, with DMH treatment.

INTRODUCTION

Colorectal cancer is a major cause of death among patients with internal malignancies in the United States and other Western countries and, unfortunately, is incurable in approximately one-half of these patients at the time of initial diagnosis (1). During the last 40 years, surgery, chemotherapy, and radiotherapy have not significantly altered cure rates for this disease (1). Based on these considerations, the search for strategies to prevent the development of colonic tumors has markedly intensified during the past few years.

In this regard, epidemiological studies have strongly suggested that environmental factors, particularly dietary factors such as fat intake, promote the development of colon cancer (2, 3). Based on these observations, it would appear that identification of these factors and their reduction in the diet may be an important and practical way to reduce cancer risk (2, 3). Alternatively, the possibility of using dietary anticarcinogens as a strategy to prevent colon cancer has also recently been recognized (3).

Vitamin D and dietary calcium are two such potential colonic anticarcinogens that have received increasing attention during the past few years. Data have accumulated for and against the possibility that these nutrients may play a protective role in colon cancer. Evidence in support of this contention comes from several different sources. A number of epidemiological studies (4–7) have suggested that dietary calcium and/or vitamin D derived from dietary sources or from cutaneous synthesis in response to sunlight exposure may reduce the risk of colorectal cancer. The active hormonal form of vitamin D3, 1,25(OH)2D3, has also been shown to inhibit the proliferation of a number of malignant cell lines in vitro (8–11), including those derived from human colonic adenocarcinomas (9–11), as well as to inhibit the promotion of chemical carcinogenesis in mouse skin (12, 13). Additionally, some studies have reported that supplemental dietary calcium reduces colonic proliferation in individuals judged to be at high risk for the development of colon cancer (14–17) and inhibits the increased colonic proliferation induced by bile acids and fatty acids in experimental animals (18–21) and human colon cell lines (22). Calcium or vitamin D supplementation have also been shown to inhibit the incidence of colon carcinogenesis induced by DMH in rats fed a high fat diet (23) as well as tumor yield in rodents treated with azoxymethane after enterectomy (24).

In contrast to these observations, international ecological data (25) as well as two recent case-control studies (2, 26) have failed to demonstrate a protective effect of calcium on colon cancer. Moreover, in certain in vitro experiments, 1,25(OH)2D3 has actually enhanced chemically induced transformation in cultured cells (27, 28). Several studies, utilizing animal colon carcinogenesis models, have failed to show an effect of calcium on the promotional effects of fat or bile acids on this malignancy (2, 29, 30). Gregoire et al. (31) reported that calcium supplementation did not decrease colonic mucosal proliferation in patients previously operated on for colon adenocarcinoma. Finally, as noted by Bresalier and Kim (32), it is important to point out that while raised colonic mucosal proliferation rates are associated with carcinogenesis, to date, suppression of proliferation per se by calcium, vitamin D, or other agents has not been proven to prevent the onset of malignancy in humans or animals.

Given these conflicting data, the possible roles of calcium and vitamin D as colonic anticarcinogenic agents remain unclear. In an attempt to clarify this important but controversial issue, in the present studies it was of interest to examine the possible role of supplementary dietary calcium alone or in conjunction with vitamin D deficiency on colonic tumorigenesis in rats utilizing the well-known procarcinogen DMH (33). In

3The abbreviations used are: DMH, 1,2-dimethylhydrazine; 1,25(OH)2D3, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ODC, ornithine decarboxylase.
addition, we have assessed the effects of these dietary manipulations on colonic mucosal levels of polyamines, compounds that have been implicated as intimately involved in the malignant transformation process of the colon in humans and experimental animals (34, 35). The results of these experiments as well as a discussion of their possible significance with respect to carcinogenesis in the colon serve as the basis of this report.

MATERIALS AND METHODS

Animals. Three groups of male albino Sprague-Dawley rats (40–46 rats/group), initially weighing 40–60 g, were used in these experiments. All diets were obtained from ICN Biochemicals, Cleveland, OH, and are described in detail in Table 1. Group A was fed a diet containing a normal content of calcium and phosphorus, 0.87 and 0.60%, respectively, with 2.2 IU of vitamin D3/g of feed. Group B was fed the same diet as Group A, but with calcium and phosphorus increased to 1.80 and 0.80%, respectively, with 2.2 IU of vitamin D3/g of feed. Group C was fed a vitamin D-deficient diet containing normal content of calcium and phosphorus, 0.87 and 0.60%, respectively, with 2.2 IU of vitamin D3/g of feed. All rats were housed in a dark room to prevent cutaneous vitamin D synthesis. A vitamin D deficient, normal calcium diet could not be studied, inasmuch as rats on this diet grow poorly and do not tolerate the high dietary calcium. Group C was fed a vitamin D-deficient diet containing 1.8% calcium and 0.80% phosphorus, which as previously shown (36) was sufficient to prevent the development of calcium deficiency, phosphate depletion, and/or secondary hyperparathyroidism, known complications of vitamin D deprivation. Animals from each group were fed their respective diets with food and water ad libitum for up to 33 weeks. All rats were housed in a dark room to prevent cutaneous vitamin D synthesis. A vitamin D deficient, normal calcium diet could not be studied, inasmuch as rats on this diet grow poorly and do not tolerate the carcinogen. At the end of 6 weeks, when the rats weighed about 200–250 g, one-half of the animals in each group were then excised and examined macroscopically and microscopically for the presence of tumors. All but one rat in dietary group C, who died of metastatic colon cancer after 25 weeks of DMH, survived until planned sacrifice.

Histological Studies. At sacrifice, all macroscopic lesions as well as tissue at least 1 cm away from these lesions from each animal in each DMH-treated group were immediately fixed in neutral buffered formalin. In the control groups, multiple samples (at least 4) were randomly taken from each animal’s colon. Fixed specimens were then embedded for light microscopic examination and stained with hematoxylin and eosin (33). All specimens were evaluated by a pathologist (C. A.) who was unaware of the dietary or drug treatment history.

Measurement of Serum Calcium, 25(OH)D, and 1,25(OH)2D Levels. Serum 25(OH)D levels were measured by a modified competitive protein-binding assay as described previously (37). The intraassay coefficient of variation was 5.3% and the interassay coefficient of variation was 11.3%. 1,25(OH)2D was assayed by a commercially available kit that uses calf thymus receptor as a binder (Catalogue No. 60065; INCSTAR Corp., Stillwater, MN). Serum calcium was measured by atomic absorption spectrophotometry.

Colonic Mucosal Polyamine Levels and Ornithine Decarboxylase Activity. Twenty-eight rats from each dietary group were treated for 15 weeks with either diluent (n = 14) or DMH (n = 14). In agreement with earlier findings from our laboratory (33), at this time point there was no evidence of severe atypia, carcinoma in situ, or invasive carcinoma by light microscopic examination in the DMH-treated rats. Only mild inflammation was observed that was similar in the three dietary groups. For measurement of colonic mucosal polyamine levels, colonic mucosal samples were rapidly scraped into 0.2 M HClO4 (approximately 1 g tissue, wet weight/10 ml acid). Samples were homogenized using a Brinkmann polytron at a setting of 4 for 60 s and then microfiltered through a 0.22-µm membrane (Millex-GV; Millipore, Bedford, MA). Aliquots of 25–100 µl of the samples were then analyzed for putrescine, spermidine, spermine, and N4-acetyl spermidine by reversed-phase, high-performance liquid chromatography according to the method of Seiler and Knodgen (38). Sample components were found to be stable when kept at 4°C for weeks or −70°C for up to 6 months.

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Table 1 Composition of experimental diets

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<th>Ingredient</th>
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<th>B (%)</th>
<th>C (%)</th>
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CALCIUM, VITAMIN D, AND COLON CANCER

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The identity of $N^2$-acetylspemidine was further confirmed by acid hydrolysis and thin-layer chromatography as described by Matsuzaki et al. (39).

For assay of ODC activity, colonic mucosal scrapings were homogenized in 50 mM Tris-HCl, 5 mM dithiothreitol buffer, pH 7.4. Homogenates were then centrifuged at 105,000 $\times$ g for 60 min and the supernatants were used as the enzyme source. Protein was determined by the method of Lowry et al. (40), using bovine serum albumin as standard. ODC was assayed by the method of Pegg and Williams-Ashman (41) as modified by Steeves and Lawson (42). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.4), 5 mM dithiothreitol, 0.2 mM pyridoxal phosphate, 0.2 $\mu$Ci l-[1^{-14}C]ornithine and 0.5-1.0 mg of protein in a final volume of 0.3 ml. The reaction was incubated at 37°C in a 16- x 100-mm glass tube sealed with a rubber stopper supporting a center well (Kontes, Morton Grove, IL). The released $^{14}$CO$_2$ was trapped on a disc of Whatman GFA glass fiber filter paper soaked with 200 $\mu$l of NCS (Amersham, Arlington Heights, IL) in the suspended center well. The reaction was stopped at 30 min by injecting 0.5 ml of 2 N HCl through the rubber stopper and it was allowed to sit at room temperature for 30 min to completely trap the released $^{14}$CO$_2$. The filter paper and center well were then removed and placed in a scintillation vial containing 2 ml of ethanol; 10 ml of Scintiverse E were added and radioactivity was measured in a Beckman LS 6800 liquid scintillation counter.

Statistical Methods. Fisher's exact test or unpaired $t$ tests were used for statistical analyses as appropriate (43). $P < 0.05$ was considered significant.

**RESULTS**

Animal Growth. As shown in Fig. 1, rats in dietary group B initially grew less than animals in the other two dietary groups. When treatment with DMH began at week 6, however, growth rates were comparable in all groups, and there were no differences in final body weights. In all three dietary groups, animals treated with DMH and those receiving vehicle had comparable growth rates (Fig. 1).

Serum Calcium and Vitamin D Metabolite Levels. Table 2 presents the serum calcium and vitamin D metabolite levels from the DMH-treated rats after 26 weeks of procarcinogen exposure. Animals in groups A and B had the same serum calcium and 25(OH)D levels. There was a trend for the calcium-supplemented rats (group B) to have lower serum 1,25(OH)$_2$D levels, but this was not statistically significant. As reported previously (36), the animals on this vitamin D-deficient, calcium-supplemented diet (group C) did not develop hypocalcemia. Serum 25(OH)D and 1,25(OH)$_2$D levels, however, were significantly lower than those seen in group B rats that received a high-calcium, vitamin D-sufficient diet. No significant differences in serum calcium and vitamin D metabolite levels were observed between DMH-treated and vehicle-treated rats in any of the three dietary groups (data not shown). After 21 weeks on the experimental diets, when the measurements of colonic mucosal polyamine levels were performed, the serum 25(OH)D levels in group C were uniformly less than 10 mg/ml, indicating that vitamin D deficiency was well established by that time.

Colon Tumors. In agreement with previous studies from our laboratory (33), none of the control animals in the three dietary groups developed macroscopic or microscopic neoplasms at the time of sacrifice. After 26 weeks of DMH exposure, nine, ten, and nine rats from groups A, B, and C, respectively, were found to have colonic neoplasms (Table 3). The majority of the tumors in each dietary group were moderately well to poorly differentiated colonic adenocarcinomas, as assessed by light microscopy. Adenomas or carcinomas in situ represented only two, three, and one tumor in groups A, B, and C, respectively. It appears, therefore, that dietary calcium supplementation and/or vitamin D nutritional status did not influence the number of animals that developed tumors secondary to DMH in the present experiments.

In contrast to these findings, however, analysis of the number of tumors per tumor-bearing rat revealed significant differences among the DMH-treated groups (Table 3). In the control dietary group (group A), 6 of 9 rats with colonic neoplasms had...
with 3 cancers. In contrast, with calcium supplementation, rats with 2 tumors and 1 animal with 4 tumors. The mean number of tumors per tumor-bearing rat was 1.8, 1.1, and 1.9 in groups A, B, and C, respectively.

Differences in tumor size were also observed in the three dietary groups. Tumors in group B rats were always small, with a greatest dimension of 6 mm or less. In contrast, 8 of 16 and 8 of 17 neoplasms in groups A and C, respectively, were greater than 6 mm. The only two rats with visible disease metastatic to mesenteric nodes and liver were in group C. With these semisynthetic diets, tumors were approximately evenly distributed between the proximal and distal colon in all groups.

Colonic Mucosal Polyamine Levels. After 15 weeks of DMH treatment, in group A, colonic mucosal levels of all four polyamines studied, putrescine, spermidine, spermine, and N\(^1\)-acetylspermidine, were significantly increased compared to vehicle-treated rats (Fig. 2). In the calcium-supplemented group, N\(^1\)-acetylspermidine was slightly higher, but the other polyamine levels were unchanged in vehicle-treated animals. In group B rats, DMH treatment caused only a small rise in putrescine content, and no change in spermidine, spermine, or N\(^1\)-acetylspermidine. In dietary group C, DMH-exposure significantly increased mucosal N\(^1\)-acetylspermidine but did not affect the other polyamines.

ODC-specific activity showed great variability, and the data were, therefore, expressed as the difference between vehicle and DMH-treated rats studied in the same assay run. As previously reported (44), in the control dietary group A, DMH treatment tended to increase ornithine decarboxylase-specific activity by 4.4 ± 3.2 (SE; n = 6/group) pmol/mg protein/h. In contrast, in both groups B and C, DMH-treated rats showed no change or even a decrease in ODC-specific activity, −3.7 ± 4.2 and −2.3 ± 5.4 pmol/mg protein/h, respectively.

**DISCUSSION**

In the present study, supplemental dietary calcium failed to influence the incidence of colon cancers induced by DMH. This observation is in basic agreement with several prior studies utilizing DMH or other carcinogens (2, 23). For example, Pence and Buddingh (23) found that supplemental calcium did not alter tumor incidence in rats fed a low-fat diet, 5.0%, as was used in the present experiments. Calcium, however, lowered tumor incidence when a high-fat diet (20%) was used to promote carcinogenesis. Wargovich et al. (45) also found that calcium decreased tumor incidence in azoxymethane-treated rats on a high fat, but not low-fat diet.

In the present experiments, calcium supplementation decreased the tumor burden, as assessed by the frequency of rats with multiple colonic neoplasms. Whereas 6 of 9 rats with tumors in the control diet group had multiple neoplasms, only 1 of 10 calcium-supplemented animals had 2 colon tumors. Similar results were obtained by Appleton et al. (24), who studied rats given azoxymethane after partial enterectomy, and found a reduction in the number of tumors per rat with dietary calcium supplementation. Using the same carcinogen, Wargovich et al. (45) reported that calcium decreased the number of adenocarcinomas but not adenomas per tumor-bearing rat. In contrast, McSherry et al. (30) found that dietary calcium supplementation did not inhibit tumor formation in rats receiving N-methyl-N-nitrosourea, as assessed by tumor incidence, tumors/rat, or tumors/tumor-bearing rat. They did note, however, that high dietary calcium reduced the number of invasive cancers. In the present study, rats on the high-calcium diet uniformly developed small neoplasms (<6 mm), and tended to have a higher percentage of adenomas and carcinomas in situ than the control group.

The effect of dietary vitamin D on experimental colon carcinogenesis has been investigated less extensively. Kawaura et al. (46) found that 1-a-hydroxyvitamin D\(_3\), which is rapidly converted in vivo to 1,25(OH)\(_2\)D\(_3\), inhibited the promotional effect of lithocholic acid in N-methyl-N-nitrosourea-induced colonic tumorigenesis in the rat. Pence and Buddingh (23) reported that supplemental vitamin D inhibited DMH-induced tumorigenesis in rats fed a high-fat but not low-fat diet. In a preliminary report, Comer et al. (47) found that supplemental vitamin D decreased the incidence of noninvasive adenocarcinomas in distal but not proximal colons in DMH-treated rats. In the present study, the group on the vitamin D-sufficient, high-calcium diet had the same tumor incidence as the vitamin D-deficient, high-calcium group but had significantly more rats with multiple neoplasms. In addition, one-half of the tumors observed in the vitamin D group were large (>6 mm), and metastatic cancers were observed only in this group. These data suggest that associated vitamin D deficiency tends to negate the protective effect of high dietary calcium.

Increased intracellular polyamine levels have been found in colonic neoplasms, and the activity of the enzyme ODC, which
converting ornithine to putrescine, has been reported to be increased in colon cancers (34, 35). Furthermore, treatment with 2-difluoromethylornithine, a specific inhibitor of ODC, inhibits DMH-induced colon tumors in mice and azoxymethane-induced colonic cancers in the rat (34, 48). Previous studies from this laboratory have demonstrated that after administration of DMH to rats for 15 weeks, before development of recognizable neoplasia, colonic mucosal ODC activity and putrescine content are increased (44). In addition, the “reverse” pathway for putrescine formation was activated in the premalignant mucosa, as mucosal N²-acetylsermidine was elevated and the specific activity of spermidine N²-acetyltransferase was increased (44).

In the present study, administration of DMH for 15 weeks caused significant rises in the mucosal polyamine content and tended to increase ODC-specific activity in the control diet group. Calcium supplementation had little effect on polyamine levels in the vehicle-treated rats but blunted the response to DMH administration. Arlow et al. (49) and Behling et al. (50) found that calcium attenuated the rise in colonic mucosal ODC induced by azoxymethane and DMH. The relationship of these observations to the beneficial effect of calcium on tumorigenesis seen in our experiments requires further study, but altered polyamine metabolism induced by calcium supplementation could play an important role.

Hashiba et al. (51) have demonstrated that 1-a-hydroxyvitamin D₃ inhibits the increase of ODC activity induced by tumor promoters, including that stimulated by deoxycholate in colonic mucosa. In the present study, animals on the vitamin D-deficient, calcium-supplemented diet demonstrated an increase in mucosal N²-acetylsermidine levels, but not other polyamines, in response to DMH administration that was not observed in rats on the vitamin D-sufficient, high-calcium diet. Since vitamin D deficiency reversed the protective effects of calcium on tumorigenesis, these data are supportive of the view that the “reverse” pathway and N²-acetylsermidine may be particularly important modulators of colonic carcinogenesis, at least in this model (44).

A number of investigators have suggested that the colonic anticanicogenic property of calcium may involve reduction of the carcinogenic/promoter effects of free fatty acids and bile acids on the colon by converting them to insoluble calcium soaps in the lumen of the large intestine (18-21). Others have suggested that calcium may have a direct effect on the colonic mucosa as well as the indirect influences noted above (22, 52).

In the present experiments, vitamin D deficiency negated the protective effects seen with calcium supplementation alone. While fecal calcium concentrations were not measured in this study, it is expected that vitamin D deficiency would lead to calcium malabsorption and, therefore, increased luminal calcium concentrations. It is well established, moreover, that vitamin D deficiency reduces colonic calcium absorption in the rat (53). These observations would suggest that colonic luminal calcium may not be as important as intracellular calcium in protecting against colon cancer, as previously hypothesized.

Alternatively, vitamin D deficiency may promote carcinogenesis by mechanisms independent of the effect of the vitamin on calcium transport. Recent studies (reviewed in Ref. 54) have shown that 1,25(OH)₂D₃ has a number of biological actions which may be important with respect to tumor cell growth and development, including influencing immune modulation, oncogene expression, signal transduction, and other processes.

In summary, the present studies indicate that dietary calcium supplementation may inhibit DMH-induced colon tumorigenesis, decreasing the number and size of neoplasms. Vitamin D deficiency abolished the protective effect of high dietary calcium. Altered polyamine metabolism is one potential mechanism by which these nutrients may influence carcinogenesis. Preliminary work in this laboratory has suggested that dietary calcium may also decrease the prevalence of K-ras oncogene mutations in DMH-induced colon cancers and that this effect is also reversed by vitamin D deficiency (55). Further studies to clarify the multiple mechanisms by which calcium and vitamin D may modulate the multistep process of colonic carcinogenesis are being conducted in our laboratory.

ACKNOWLEDGMENTS

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