Inadequate dietary copper increases tumorigenesis in the Min mouse

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Received 24 April 2000; received in revised form 21 June 2000; accepted 26 June 2000

Abstract

Multiple intestinal neoplasia (Min) mice are a good model for the investigation of the effects of dietary alterations on genetic susceptibility for intestinal cancer. In the current study, nursing dams and their pups were placed on an AIN-93G diet containing either 1 or 6 ppm copper. The pups were maintained on the same concentration of dietary copper after weaning until they were 13-weeks-old. Animals fed copper deficient diets had a significantly ($P < 0.0003$) higher small intestine tumor incidence and a significantly ($P < 0.04$) higher small intestine tumor burden than animals fed adequate dietary copper. Therefore, inadequate dietary copper can increase the spontaneous tumorigenesis that occurs in the Min mouse. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Min mice; Copper; Intestinal cancer

1. Introduction

Colon cancer is the second leading cause of cancer mortality in the United States and the fourth most common cause of cancer mortality worldwide [1]. It is believed that diet is the single greatest contributor to this human cancer, possibly accounting for 35–45% of the disease [2]. One possible dietary factor that may increase the susceptibility to colon cancer is inadequate dietary copper. Recent studies [3,4] have shown that ingestion of a diet deficient in copper significantly increased the formation of 3,2'-dimethyl-4-aminobiphenyl and dimethyl hydrazine-induced aberrant crypt foci in rats. Aberrant crypt foci are preneoplastic lesions that have been detected in human colon resections and in experimental animals treated with chemical carcinogens [5,6]. Studies in humans have suggested that colonic aberrant crypt foci are precursor lesions from which adenomas and adenocarcinomas will develop [7,8]. Dietary factors are widely accepted as risk factors for colorectal cancer. A number of natural compounds and nutrients that inhibit carcinogen-induced aberrant crypt foci development have been proven to have chemopreventive activity against colon cancer in rodents [9]. Two studies have shown that copper deficiency increased the incidence of chemically-induced colon cancer in experimental
animals [10,11]. Thus, low dietary copper may be a potential risk factor for colon cancer in humans.

The Food and Nutrition Board estimates that the estimated safe and adequate daily intake of dietary copper is 1.5–3.0 mg for adults [12]. The Western diet frequently is low in copper in comparison with suggested standards [13]. Calculations based on surveys of 849 individuals from North America and Europe, in which copper content was measured by chemical analysis, indicated that 61% of the diets provided <1.5 mg Cu/day and approximately one-third are <1 mg Cu/day [14]. Furthermore, surveyed hospital diets provided intakes of only 0.62–0.86 mg Cu/day [15]. Thus, inadequate copper intake is a practical concern in the US population.

Both human and animal studies suggest that mutation of the tumor suppressor gene adenomatous polyposis coli (APC) is a powerful facilitator of intestinal tumorigenesis. Germline mutations of this autosomal dominant gene lead to familial adenomatous polyposis (FAP), a disorder characterized by an early development of multiple adenomas of the colorectum and duodenum with progression to colorectal carcinoma in the third to fourth decade of life in an untreated individual [16,17]. Although FAP patients with germline mutations of APC account for less than 1% of colorectal cancer in the United States, somatic mutations of the APC gene occur in the vast majority of sporadic colorectal cancers [18–20]. Such alterations can be found in the smallest lesions examined, such as aberrant crypt foci, suggesting that they are an early event in colorectal tumorigenesis [21,22].

A role for APC in colon carcinogenesis has been further corroborated by the discovery of mouse models of FAP. These models include the multiple intestinal neoplasia (Min) mouse, which has a nonsense mutation in codon 850 of the murine APC gene, which is a homolog of the human APC gene [23,24]. These mice are highly susceptible to spontaneous formation of numerous tumors in both the small and large intestine [23,24]. The Min mouse presents an opportunity to study the pathogenesis of a neoplasia in which the initial genetic defect is the same between human and mouse. The purpose of the current study was to investigate the relationship between dietary copper and intestinal cancer susceptibility in Min mice.

2. Materials and methods

Breeding pairs of C57BL/6J +/+ (wildtype) females and C57BL/6J-Min/+ males were purchased from the Jackson Laboratory (Bar Harbor, ME). The breeding pairs were fed an AIN-93G diet [25], until their pups reached 1-week-old. At that time, the nursing dams and their pups were placed on an AIN-93G diet containing either 1 or 6 ppm copper (by analysis, 0.76 and 5.49 μg Cu/g diet, respectively). Animals were provided free access to demineralized water and purified diet. When the pups were weaned, they were genotyped. The Min/+ mice were identified by allele-specific polymerase chain reaction (PCR) [26,27]. The wildtype (+/+ ) littermates were sacrificed. The Min/+ mouse pups were separated according to their sex (n = 6 females and n = 4 males when diet copper = 1 ppm; n = 4 females and n = 7 males when diet copper = 6 ppm) and continued on the same diet until the end of week 13 when the experiment was terminated. Thirteen weeks was chosen because most Min mice develop chronic anemia by 60 days of age and it usually causes death by 120 days of age in most affected mice. Dietary copper deficiency will exacerbate this anemia.

Animals were fasted overnight prior to sacrifice. After sacrifice, the entire small and large intestines were removed, opened, spread out with the lumen side up and fixed in 10% neutral buffered formalin. The small intestine was divided into three sections of equal length, namely the proximal, middle and distal sections, while the large intestine was not divided. Visible tumors along the entire length of the small and large intestines were counted and measured under a stereo-microscope at a magnification of x20. The fixed colon and rectum were stained with 0.1% methylene blue in 0.1 mol/l sodium phosphate buffer and analyzed for aberrant crypts. An aberrant crypt foci was defined as one or more aberrant crypts in a cluster.

Samples of liver and colon were analyzed for copper, iron and zinc by inductively coupled argon atomic emission spectrometry (Liberty Series II, Varian Associates, Sugarland, TX). Briefly, the tissues were weighed, lyophilized to constant weight and wet ashed multiple times with nitric acid until most of the organic residue was gone. The charred samples were dissolved in 3 ml nitric acid and 10 ml hydrogen perox-
ide and heated to dryness on a hotplate. The mineral residue was dissolved in 1 ml of 6 mol/l HCl and diluted appropriately with deionized water. Liver standard reference material (1577b, National Institute of Standards and Technology, Gaithersburg, MD) was analyzed with each batch of tissue samples for quality control. Liver standard samples \((n = 4)\) were determined to contain 99.0, 104.4 and 101.4% of the certified values for copper, iron and zinc, respectively.

### 3. Results

Dietary copper did not affect the weight gain of mice. However, male mice had significantly \((P < 0.0001, \text{2-way ANOVA})\) greater body weights at all time points than female mice.

Animals fed the copper deficient diets had a significantly \((P < 0.0003, \text{Kruskal–Wallis test})\) higher small intestine tumor incidence (Fig. 1) and a significantly \((P < 0.04, \text{Kruskal–Wallis test})\) greater small intestine total tumor burden (Fig. 2) than animals fed adequate dietary copper. Gender did not affect the rate of tumor development. Only a few tumors were found in the large intestine (Table 1) and no statistically significant effects of dietary copper were observed on the incidence, number, or mass of tumors in the large intestine. Only a few aberrant crypts were observed in the large intestine (Table 2). No statistically significant effects of dietary copper were observed on the incidence, number of aberrant crypt foci or total number of aberrant crypts.

Animals fed deficient dietary copper had significantly \((P < 0.005, \text{Students} t\text{-test})\) depressed liver and colon copper concentrations relative to adequate dietary copper (Table 3). Dietary copper had no significant effects on liver or colon iron or zinc concentrations.

### 4. Discussion

The current study demonstrates that dietary copper deficiency increases the incidence of spontaneous

Fig. 1. Total number of tumors in the small intestine of Min mice fed deficient or adequate concentrations of dietary copper (by analysis, 0.76 and 5.49 \(\mu\text{g Cu/g diet}\)). Values are means ± SEM, \(n = 10\) or 11. Animals fed the copper deficient diets had significantly \((P < 0.0003, \text{Kruskal–Wallis test})\) higher tumor number than animals fed adequate dietary copper.

![Graph showing total number of tumors in the small intestine of Min mice fed deficient or adequate concentrations of dietary copper.](image)

Table 1

<table>
<thead>
<tr>
<th>Diet copper</th>
<th>Incidence</th>
<th>Tumor #</th>
<th>Tumor burden (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/10</td>
<td>1.00 ± 0.45</td>
<td>1.88 ± 0.86</td>
</tr>
<tr>
<td>6</td>
<td>2/11</td>
<td>0.27 ± 0.19</td>
<td>0.21 ± 0.15</td>
</tr>
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</table>

\(a\) Number of mice with tumors/number of mice in the group.

\(b\) Values are mean ± SEM, \(n = 10\) or 11.
tumorigenesis that occurs in the Min mouse. Our results are similar to other investigators who have observed that in Min mice most of the tumors usually occur in the small intestine rather than in the large intestine [28]. However, because of the genetic and histochemical similarities between small intestinal tumors in the Min mouse and colon cancer in humans, these mice are an accepted model for colon cancer in humans [28]. Our results parallel studies which demonstrate that dietary copper deficiency can increase the formation of chemically (3,2'-dimethyl-4-aminobiphenyl and dimethyl hydrazine)-induced aberrant crypt formation. Although the exact mechanism for the protective effect of dietary copper against colon tumorigenesis is unknown, one potential mechanism is alterations in antioxidant enzymes. Two copper containing enzymes, namely copper–zinc superoxide dismutase and ceruloplasmin, that may help protect against oxygen radical-mediated injury are significantly reduced in animals fed low copper diets [3,4]. Copper–zinc superoxide dismutase functions to eliminate superoxide radicals and ceruloplasmin is hypothesized to inhibit iron-catalyzed radical formation [29,30]. Substantial evidence has suggested that free radicals, particularly oxygen radicals, are involved in both the initiation and promotion stages of carcinogenesis [31]. Much of the evidence relies on the fact that antioxidants that scavenge free radicals directly, or that interfere with the generation of free radical-mediated events, reduce the incidence of neoplasia and that the activities of antioxidant enzymes are altered in tumor tissue [31]. For example, when compared with their normal cell counterparts, tumor cells are always low in manganese superoxide dismutase and usually low in copper–zinc superoxide dismutase activity [31–35].

Another potential mechanism for the protective effect of dietary copper against colon carcinogenesis may be alterations in protein kinase C expression. Reduced total PKC activity has been detected in primary human and rat colonic neoplasms compared with paired adjacent normal colonic mucosa [36–39]. Although protein kinase C is not a cuproenzyme, dietary copper deficiency has been shown to cause alterations in protein kinase C activity and isoform distribution [4,40]. We recently observed that low dietary copper increased dimethylhydrazine-induced aberrant crypt formation and decreased protein kinase Cα, δ, and ξ protein expression. Thus, changes in protein kinase C isoform protein expression may be related to increased susceptibility of copper-deficient animals to intestinal cancer. Future studies will investigate whether changes in protein kinase C isoform expression and localization are associated with the protective effect of dietary copper against tumorigenesis in the Min mouse.

In conclusion, our results demonstrate that inadequate dietary copper can increase the spontaneous tumorigenesis that occurs in the Min mouse. These results have practical implications because more

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<th>Aberrant crypt focib</th>
<th>Aberrant crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/10</td>
<td>0.90 ± 0.46</td>
<td>1.40 ± 0.60</td>
</tr>
<tr>
<td>6</td>
<td>1/11</td>
<td>0.09 ± 0.09</td>
<td>0.09 ± 0.09</td>
</tr>
</tbody>
</table>

a Number of mice with aberrant crypt foci/number of mice in the group.
b Values are means ± SEM, n = 10 or 11.

Table 3
Effect of dietary copper on liver and colon mineral concentrations

<table>
<thead>
<tr>
<th>Diet copper</th>
<th>Liver</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Copper (μmol/g dry weight)a</td>
<td>Iron (μmol/g dry weight)</td>
</tr>
<tr>
<td>1</td>
<td>0.218 ± 0.008b</td>
<td>0.621 ± 0.125</td>
</tr>
<tr>
<td>6</td>
<td>0.313 ± 0.015</td>
<td>0.593 ± 0.095</td>
</tr>
</tbody>
</table>

a Values are means ± SEM, n = 11.
b Significant effect of dietary copper (P < 0.005, Students t-test)
than 60% of the diets consumed in the US do not contain the recommended amount of copper.

Acknowledgements

The authors would like to thank Laura Idso and Tiffany Carbaugh for technical assistance. The authors would also like to thank Denice Schafer and her staff for mixing the diets, taking care of the animals, and collecting blood for genotype analysis.

References