Tissue zinc levels in precancerous tissue in the gastrointestinal tract of azoxymethane (AOM)-treated rats

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Received 23 April 2007; accepted 8 October 2007

Abstract

Alterations in tissue zinc levels have been documented in patients with gastrointestinal tract malignancies and more frequently, in those with colonic cancer. However, the precise role of tissue zinc in carcinogenesis is not well elucidated.

This study, using a well-established colon cancer model in rats, was designed to investigate the relationship of tissue zinc to the carcinogenic process. The aim was to examine tissue zinc levels in the preneoplastic tissues and to study the changes that occur during transition of mucosa from normal to preneoplastic state.

Six-week old rats were given a single dose subcutaneous injection of azoxymethane (AOM) (30 mg/kg body weight) and sacrificed after 1, 2, 5, and 9 months of the treatment. Plasma zinc levels showed a significant decrease ($p < 0.05$) at 9 months compared with controls. Tissue zinc levels showed a significant decrease in the large intestine at 1 and 2 months ($p < 0.05$) and at 5 and 9 months ($p < 0.01$), in the small intestine at 2, 5, and 9 months ($p < 0.05$), and in the stomach at 5 and 9 months ($p < 0.05$). The maximum percent decrease (45%) in tissue zinc was observed in the large intestine at 9 months. Tissue copper zinc super oxide dismutase (CuZnSOD) activity was assessed in the body of the stomach, small intestine, and large intestine and compared with the control group. There was a significant fall in CuZnSOD levels in the small intestine at 9 months ($p < 0.05$) and in the large intestine at 5 and 9 months ($p < 0.01$). Two of these six rats showed histological evidence of precancerous lesions in the mucosa of the colon. This study suggests that the decrease in plasma zinc, tissue zinc and activity of CuZnSOD is associated with development of preneoplastic lesions in the colonic mucosa.

Keywords: Colon carcinoma; Azoxymethane; Zinc; CuZnSOD; Precancerous

Introduction

In the developed countries, the colon is a common site for malignancies of the gastrointestinal tract, which are also a leading cause of death. Many contributing factors such as lifestyle, nutrition and obesity have been
postulated to play a role in the development of these malignancies (Mohandas and Desai, 1999).

Zinc is necessary for the functioning of all living systems. It is an essential trace element, important for the stabilization and function of numerous metalloenzymes involved in protein synthesis, protein catabolism, energy metabolism, and RNA and DNA synthesis (Sky Peck, 1986). Since zinc was first reported as a constituent of carbonic anhydrase, in 1940, it has now been identified as a component of more than 200 mammalian metalloenzymes. These metalloenzymes are activated by zinc incorporation. Zinc plays an important role in protein metabolism in humans and is necessary for maintenance of normal levels of proteins. The role of zinc in RNA, DNA polymerases, its inhibitory effects on phosphodies- trases and its activating effect on membrane-bound adeny cyclase; all suggest a role for zinc in carcinogenesis (Vallee and Goldes, 1984). It is well established that zinc plays an essential role in a number of biological processes, through its action as an activator or inhibitor of enzymatic reactions, by competing with other elements and proteins for binding sites, and influencing permeability of the cell membrane. It is therefore reasonable to assume that zinc could exert an action directly or indirectly on the carcinogenic process (Sunderman, 1984).

The role of zinc in carcinoma development has been the subject of debate, and reports of zinc values in the various forms of cancer (Valcovic, 1980). Low plasma zinc levels have been observed in patients with cancer of the colon, bronchus, and digestive system (Karcicoglu et al., 1980). Ehud et al. showed a decrease in tissue zinc levels in large bowel cancer and stomach cancer compared with normal tissues (Ehud et al., 1983). The mechanism by which serum zinc and tissue zinc decrease in various cancerous tissues is still obscure; neither has it yet been established, whether these altered zinc levels contribute to the malignant state.

Oxidative stress caused by reactive oxygen metabolites, result in damage to cellular structure, and this has frequently been implicated in the initiation and promotion phases of carcinogenesis. Super oxide dismutases (SOD) are metalloenzymes that play a vital role in the protection of aerobic cells against oxygen toxicity and cytosolic CuZnSOD has been shown to contain both copper and zinc atoms (Cindy and Davis, 1999). Superoxide anion (O$_2^-$) is a free radical, produced by partial reduction of molecular oxygen in several metabolic processes. In the presence of two molecules of hydrogen gas, two molecules of oxygen are converted to H$_2$O and O$_2$. The rate of this reaction is greatly increased by SOD. This enzyme, which is essentially absent from anaerobic cells, is important as a scavenger of oxygen-free radicals that otherwise would lead to damage of the cell membrane and biological structure (Jansen and Bosman, 2000).

Tissue CuZnSOD activity is reduced in a number of malignancies. However, in other malignancies variable findings have been observed. Some tumors have less CuZnSOD in comparison with the more metabolically active tissues (Grigolo et al., 1998). The SOD plays an important role in the body’s defense mechanisms against the deleterious effects of O$_2$ free radicals in biological systems. Study by Jansen and Bosman (2000) showed that SOD was decreased in gastric malignant tissues compared with normal tissues. These findings led to the hypothesis that early mucosal alterations, which are preneoplastic lesions, might lead to a decrease in tissue zinc concentration and zinc-related enzyme CuZnSOD activity in the large intestine and to possible changes in other parts of the gastrointestinal tract.

The DNA alkylating agent azoxymethane (AOM), which is primarily activated in the liver, induces a high incidence of initiation and promotion steps of precancerous lesions in the colon of rats (Guda et al., 2003). Numerous studies have been carried out to investigate the protective effect of various chemicals, drugs and food items on the induction and development of azoxymethane-induced colon tumors in rats (Davies et al., 1999; Tao and Pereira, 1997; Seraj et al., 1997). However, so far, no studies have been reported on the tissue zinc levels and activities of zinc-containing enzymes in AOM induced colon carcinomas.

Decrease in SOD activity and in tissue zinc levels have been linked to malignancy in patients. In order to investigate their relevance in the development of the precancerous process in the colon, we have used a well-established AOM colon carcinoma model in the rat.

**Materials and methods**

**Animals**

Six-week-old adult Wistar rats (100–120 g) obtained from the institutional animal house were housed in polypropylene plastic cages, in an animal-holding room under controlled conditions with 25±2 °C, 50±10% humidity, and 12 h light–dark cycles. The rats were allowed water and food ad libitum, observed daily and weighed weekly. This study was approved by the animal experimentation ethics committee of our institution.

**Chemicals**

AOM, stock Zn standard (1002 μg/ml), bovine serum albumin, triton X100, bathocuproindisulfonate sodium
Experimental design

Forty-eight rats were randomly assigned to 8 groups of 6 each. All groups were fed the same diet. Groups 1–4 were experimental groups which received one single s.c. dose of 30 mg/kg body weight, of AOM, and were euthanized after 1, 2, 5, and 9 months, respectively. Groups 5–8 were control groups which received one single s.c. dose of 0.25 ml of saline and were euthanized after 1, 2, 5, and 9 months, respectively. All groups were euthanized by chloroform inhalation.

Tissue preparation, histopathology, measurement of tissue zinc, CuZn SOD, plasma zinc

At autopsy, the colon, small intestine and stomach of each rat were divided into 3 portions. One part of each tissue was fixed in 10% buffered formaldehyde and processed for histology study as per the standard methods. A second portion was used for tissue zinc estimation as described (Kahnke, 1966). The mucosa of the stomach, small intestine, and large intestine were used for cytosolic homogenate preparation using phosphate-buffered saline pH 7.4. CuZnSOD in the homogenate was measured by MTT reduction as described by (Kim et al., 2000).

Plasma zinc was estimated as described (Rosner and Garfien, 1968). Zinc determination was carried out using Perkin-Elmer AAS model-100. A quality control for tissue zinc estimation was described (Kahnke, 1966). The mucosa of the stomach, small intestine, and large intestine were used for cytosolic homogenate preparation using phosphate-buffered saline pH 7.4. CuZnSOD activity in the homogenate was measured by MTT reduction as described by (Kim et al., 2000).

Quality control for tissue zinc, tissue CuZnSOD, plasma zinc

The small intestine from a normal rat was cut into many pieces (30 mg each), each placed into polypropylene storage tubes and processed for tissue zinc with every batch of specimens. Ethanediol-stabilized QC serum, prepared in our laboratory, was run along with each batch of plasma zinc estimations. Similarly, the mucosa from the small intestine of a normal rat was aliquoted into storage tubes and included with each batch for estimation of CuZnSOD activity.

Statistical analysis

Data are expressed as mean ± SD. Differences between groups were analyzed using the Mann–Whitney U test and the ANOVA statistics programme. A difference was considered statistically significant when the probability associated with it was less than 0.05 (p < 0.05).

Results

Histological evidence of precancerous lesions in the mucosa of the rat colon was observed at 9 months in animals that were treated with AOM. No dysplasia occurred in the control group of rats that received saline but no AOM. Histological studies of the large intestine of rats after 9 months of treatment with AOM showed the wall of colon to have thickened mucosa, proliferated glands lined by cells with hyperchromatic pseudo-stratified nuclei and cytoplasmic–mucin depletion consistent with dysplasia as shown in Fig. 1.

Plasma zinc levels in AOM-treated rats showed a gradual decrease between 2 and 9 months with a significant decrease at 9 months compared with controls (p < 0.05) (Fig. 2).

As shown in Fig. 3a, tissue zinc levels in the large intestine showed a significant decrease (p < 0.05) at 1 and 2 months of AOM treatment with a maximum decrease at 5 and 9 months (p < 0.01) compared with controls. CuZnSOD activity in the mucosal homogenate of the large intestine showed a significant decrease at 5 months (p < 0.05) of AOM treatment, with a maximum decrease (p < 0.01) at 9 months compared with controls (Fig. 4a).

In the small intestine, a significant decrease (p < 0.05) in tissue zinc was observed at 2, 5, and 9 months compared with controls (Fig. 3b). CuZnSOD activity of the small intestine showed no changes (p > 0.05) at 1, 2, and 5 months, whereas at 9 months a significant decrease in CuZnSOD activity (p < 0.05) was observed (Fig. 4b). The histology of the small intestine showed normal morphology in the AOM-treated rats at 9 months. There was a significant decrease (p < 0.05) in tissue zinc levels in the stomach at 5 and 9 months (Fig. 3c), but CuZn SOD activity showed no changes in the body of the stomach at various periods (Fig. 4c).

Quality control (QC) values for tissue zinc, plasma zinc and tissue SOD are presented below. QC values for plasma zinc using a commercial assayed chemistry control, (Biorad, UK) are: n = 36, mean = 61 μg/dl, %CV = 7.5. Using our in-house QC, n = 24, mean = 97 μg/dl, %CV = 2.7.

Using normal rat tissue QC for tissue zinc estimation: n = 38, mean = 222 μg/g dry wt; %CV = 6.6. QC for CuZnSOD activity: n = 10, Mean = 4.68 units/g protein, %CV = 1.5.
Discussion

Initiation of mucosal changes which progress to preneoplastic lesions, may be necessary in the triggering of the adenoma—carcinoma sequence (Sandforth et al., 1988). The numerous studies on zinc levels in tissue and serum of patients with malignant tumors have come to different conclusions. A major reason for these discrepancies may be the difficulty in analyzing trace elements and the problems that exist in collecting specimens without contamination. We have made use of Atomic Absorption Spectrometry as a highly sensitive and specific technique to rapidly and accurately estimate zinc in microgram levels in a biological sample (Prasad et al., 1965).

The current study, in AOM-induced colon carcinomas in the rat model, examined the relationship between tissue zinc levels and CuZnSOD activity in the progression to precancerous stage.

After administration of a single dose of AOM, a progressive decrease of tissue zinc concentration in the stomach, small intestine and large intestine of the rat was observed at 1–9 months. A maximum decrease of 45% in tissue zinc levels was observed in the large intestine at 9 months compared with controls. In these tissues, there was also an associated mucosal change from normal to precancerous transformation. Histologically severe dysplasia was observed in the colon in 2 out of 6 rats at 9 months. In these AOM-treated rats, decreasing plasma zinc levels were also observed over a period of 9 months. These findings support the view that decreasing tissue zinc levels are representative of biochemical alterations in the colonic mucosa, which may in turn reflect the cellular changes that progressed to dysplasia and frank malignancy. The exact cause of low zinc levels in malignant tissue is not yet known. It may be due to rapid cellular division or greater utilization of zinc in cells. There appears to be a considerable agreement between our findings with respect to differences in normal—malignant tissue zinc levels in rats and studies in patients (Inutska and Araki, 1978; Ehud et al., 1983).

It has been documented that significant oxidative stress, caused by free radicals, occurs in carcinoma of the intestinal mucosa. In particular, the super oxide radical ion has been postulated as the possible cause of cancer (Oberley and Buttnor, 1979). CuZnSOD is a free-radical scavenger and hence our time course study was carried out to examine the CuZnSOD enzyme activity in the stomach, small intestine, and large intestine. This study revealed a decrease in CuZnSOD activity in the large intestine at 5 months of AOM treatment, which was pronounced by 9 months, when precancerous stage was histologically confirmed. This data suggest that CuZnSOD activity is impaired in the precancerous colonic tissue. The decreased concentration of the trace element zinc found in the precancerous tissue may...
contribute to the differences in observed tissue CuZn-SOD enzyme activities.

AOM-induced histological differentiation in the colon was directly related to a decrease in concentration of plasma zinc, tissue zinc and reduced zinc-related CuZnSOD enzyme activity. These may be sequentially related to the biochemical events in the large intestine in AOM-treated rats. In this study, the trend towards a gradual decrease in zinc concentration in the malignant tissue is in agreement with the results observed in cancer patients by Brown et al. (1980). Studies have shown that tumors develop in various regions of the gastrointestinal tract and small bowel using the AOM colon cancer model (Kristinson et al. 2002). The present study showed a decrease in tissue zinc levels in various parts of the gastrointestinal tract over a 9-month observation period. Transmural changes along the wall of the gastrointestinal tract could be the cause of this change.

In conclusion, this study indicates that there is a change in tissue zinc concentration and CuZnSOD activity, which occurs alongside the changes in the mucosa during precancerous transformation. The most

Fig. 3. Tissue zinc levels in AOM-treated rats and controls at 1, 2, 5, and 9 months. (a) Large intestine, (b) small intestine, (c) stomach. The assay was done as described in the text. Values represent mean ± SD from 6 rats. *p < 0.05, **p < 0.01, when compared with controls.

Fig. 4. Tissue CuZnSOD activities in AOM treated rats at 1, 2, 5, and 9 months. (a) large intestine, (b) small intestine, (c) stomach. The assay was done as described. Values represent mean ± SD from 6 rats. *p < 0.05, **p < 0.01 when compared with controls.
significant change was observed in the large intestine at 9 months after AOM induction, and the histology shows a progression of precancerous lesion in the colon with 2 out of 6 rats developing an obvious precancerous lesion. The animal model described in the present study is suitable for further studies on many aspects of tumor development in the colon and gastrointestinal tract. The exact mechanisms by which tissue zinc and its related enzyme CuZnSOD decrease in colon carcinomas remain to be elucidated. Additional studies are warranted to identify the mechanisms by which progression of mucosa from normal to a precancerous stage results in significant alteration in tissue zinc concentration and CuZnSOD activity in large intestine.

Acknowledgments

One of the authors (PC) thanks the Christian Medical College-Fluid Research Grant for their financial support towards this project. She also thanks Dr. Dhayakani Selvakumar and Dr. Premila Abraham for their support and guidance in preparation of this manuscript.

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