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SUPEROXIDE DISMUTASE ACTIVITY DURING DIMETHYLHYDRAZINE COLON CARCINOGENESIS AND THE EFFECTS OF CHOLIC ACID AND INDOLE

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Copper, zinc superoxide dismutase (Cu,ZnSOD) and manganese superoxide dismutase (MnSOD) activities were measured in mouse large intestinal mucosa during dimethylhydrazine (DMH) carcinogenesis. Mice were divided into five groups. Group A was subcutaneously injected with DMH (20 mg/kg) weekly and fed with a diet containing 0.2% cholic acid (C) and 0.8% indole (I). Group B was injected with DMH and given indole feeding. Group C was treated with DMH injection and cholic acid feeding. Group D was given DMH injection alone. Group E was an age-matched control group given 0.9% NaCl injection. The experiment last 21 weeks. The Cu, ZnSOD activity of intestinal mucosa in group A animals began to increase significantly at the 7th week of the experiment. In groups B, C and D, however, this enzyme was not elevated statistically until the 16th week, and then each of these groups kept an increased Cu,ZnSOD level the rest of the experimental period. MnSOD activity was elevated statistically in group C animals at the 7th week. The enzyme activity in group A and D animals increased at the 9th week, but the enzyme activity did not increase statistically until the 11th week in group B. After the 16th week of the experiment the increased activity of MnSOD in all experimental groups returned to the level of the control group. Large intestinal cancer tissues had increased Cu,ZnSOD activity and decreased MnSOD activity.

KEY WORDS: Enzyme activity, large intestinal mucosa, Cu, ZnSOD, MnSOD.

INTRODUCTION

Two forms of SOD are present in mammalian cells. A copper and zinc-containing form (Cu,ZnSOD) is found primarily in the cytoplasm and a manganese-containing form (MnSOD) is located mainly in the mitochondria. SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen. The enzyme protects cells against superoxide related toxicity.[1,2] Changes in SOD activities are observed in tumor cells. Usually tumor cells have lowered levels of MnSOD activity. Lowered amounts of Cu,ZnSOD activity have also been found in many, but not all, tumors.[3,4] Much work has been done to determine SOD activities of tumor tissues in animals[5–7] and in humans.[8–10] However, there are few observations on SOD activities during chemical carcinogenesis. In the present study we used DMH, a specific large intestine carcinogen and free radical producer, to induce large intestinal cancer in mice[11,12] and observed the changes of SOD activities with time.

High morbidity and mortality from large intestinal cancer are found in Western

people with high fat, high protein diets. This kind of diet can increase the concentration of bile acids such as cholic acid in the gut. Bile acids have been shown to be one of the promoters of large intestinal cancer and can induce the increase of reactive oxygen species.[13,14] Chung *et al.* reported that an all meat diet could increase the concentration of tryptophan and activity of tryptophanase in the gut; they postulated that indole, the product of the enzymatic action of tryptophanase on tryptophan, might be one of the factors in the etiology of colon cancer.[15] We fed mice with cholic acid and indole in the diet during DMH large intestine carcinogenesis and observed whether there was interaction between them on the formation of large intestinal cancer.

MATERIALS AND METHODS

DMH was purchased from Aldrich Chemical Co., cholic acid was from Fluka, Swiss, xanthine was from Sigma, bovine serum albumin was from Serva, xanthine oxidase was from Boehringer Mannheim GmbH. Bovine liver Cu,ZnSOD was from Diagnostic Data, Mountain View, California; porcine erythrocyte Cu,ZnSOD was from Shuchou Biochemical Reagent Co., China. Indole, nitroblue tetrazolium (NBT) were from Dongfong biochemical reagent factory, Shanghai, China. MnSOD was purified by the method of Weisiger and Fridovich,[16] with slight modification.

The female mice (Kunming strain, China, raised in Zhejiang Medical University) aged six weeks old at the beginning of the experiment were divided into five groups. Animals in group A were subcutaneously injected with DMH weekly at a dose of 20 mg DMH/kg bw and fed with a diet containig 0.2% cholic acid and 0.8% indole.[11,17,18] Group B was injected with DMH and given indole feeding. Group C was treated with DMH injection and cholic acid feeding. Group D was given DMH injection alone. Group E was an age-matched control group given a 0.9% NaCl injection. The experiment lasted 21 weeks. For another experiment, seven mice were injected with DMH alone for one year at a dose of 20 mg/kg bw.

At the 5th, 7th, 9th, 11th, 16th and 21st week of the experimental period, five or six animals in each group were killed by cervical dislocation between 1 and 3 p.m. The large intestine was immediately excised, slit lengthwise and washed in ice-cold double-distilled water to remove the lumen contents. The mucosa of the large intestine was scraped off gently with a razor blade and homogenized with 2 ml ice-cold double-distilled water for 2 min. in a glass homogenizer at 4°C.[19] The homogenate was then centrifuged at 23,000 × g for 15 min.[7] and the supernatant was kept for SOD and protein assay.

The SOD activity was measured by the inhibition of NBT reduction as described by Yamanaka *et al.*[20] with slight modification. Each assay tube contained 0.1 mM xanthine, 0.1 mM EDTA, 150 μg bovine serum albumin, 40 mM sodium carbonate, 25 μM NBT, 9.9×10^{-9} M xanthine oxidase and sample solution (5–10 μg/protein/ml) or pure SOD solution. The final volume was 3 ml. The reaction was begun by adding xanthine oxidase. After 20 min. incubation at 25°C, pH 10.2, the reaction was terminated by the addition of 1 ml of 0.8 mM $CuCl_2$ and the production of formazan was determined spectrophotometrically at 560 nm. In this assay condition, the absorbance at 560 nm of the sample without SOD was about 0.25. MnSOD activity was determined by the addition of 5 mM NaCN to the assay tube and preincubation for

30 min. to inhibit Cu,ZnSOD. For Cu,ZnSOD activity assay, crude tissue homogenate was mixed with an ethanol-chloroform mixture (0.25 and 0.15 volumes, respectively) in a XW-80 Vortex Mixer (from Shanghai Medical University) for 2 min., then centrifuged for 15 min. at 17,000 × g. The aqueous phase was saved for assay.[5] The entire procedure was done at 4°C. One unit of SOD was defined as the amount of protein that resulted in 50% inhibition of the rate of NBT reduction. Results were expressed as units per milligram protein.

Protein was determined by the method of Lowry *et al.*,[21] with the use of bovine serum albumin as the standard. Statistical significance was tested by analysis of variance with 95% significance.

For light microscopy, tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin. For electron microscopy, tissue was fixed in cold 2.5% pentadialdehyde phosphate buffer and then in 1.33% osmium tetroxide, dehydrated in ethanol and embedded. Sections of tissue 1 μm thick were stained and examined in a Philips EM 410 electron microscope.

RESULTS

In order to validate our assay, the sensitivity toward bovine liver Cu,ZnSOD (Orgotein, Diagnostic Data Inc) and chicken liver MnSOD was determined. It was found that with pure bovine liver Cu,ZnSOD, 1 unit of activity was expressed by 30 ng of protein, while with our purified chicken liver MnSOD, 1 unit of activity was expressed by 500 ng of protein. Thus, our assay is adequate to measure both Cu,ZnSOD and MnSOD.

To verify the action of DMH on large intestine carcinogenesis, we observed the morphological changes in the large intestine at the 7th, 9th, 11th, 16th and 21st week of the experimental period. The results showed that the anaplasia grade I of colon mucosa was not seen until the 16th week in each experimental group. At the 21st week of the experimental period, intestinal tumors were observed visually in animals given DMH injection and cholic acid feeding. The presence of tumor cells were verified under the light and electron microscope. In group A, however, no large intestinal tumor cells were found. Instead, the anaplasia grade II to III of colon mucosa was seen. This was also found in groups B and D (See Figs. 1–4).

Table 1 shows the temporal changes of SOD activities during DMH large intestine carcinogenesis. The Cu,ZnSOD activity of the intestinal mucosa in group A animals began to increase significantly at the 7th week with an average elevated level of 30% ($p < 0.05$). Subsequently, the enzyme activity continued to increase and showed a 62% and 57% increase at the 9th and 11th week, respectively ($p < 0.01$). At the 16th week, the enzyme activity in each experimental group had a statistically higher level than that in the control group. This higher activity lasted until the end of the experiment. The results indicated that DMH could increase Cu,ZnSOD activity during carcinogenesis and that the addition of cholic acid plus indole caused the changes to occur earlier. However, we did not observe any earlier changes in Cu,ZnSOD activity with DMH plus cholic acid or indole alone.

The MnSOD activity of intestinal mucosa in group C animals was increased significantly by the 7th week. The enzyme activity in group A and D animals increased

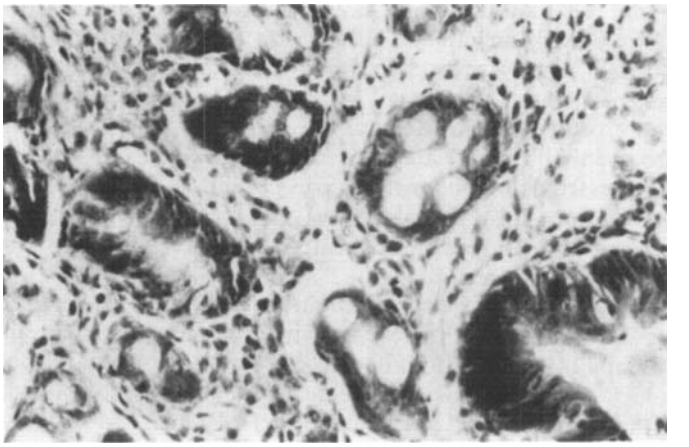

FIGURE 1 Mucosa of the distal colon at the 16th week of DMH treatment in group C. Focal hyperplastic changes can be seen (anaplasia grade I). Magnification is × 400.

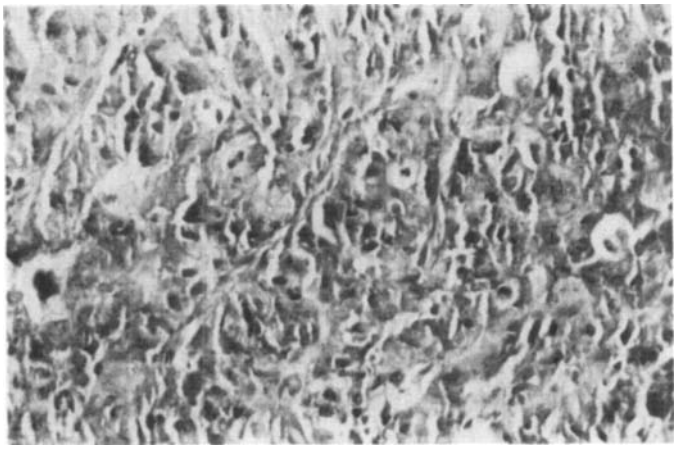

FIGURE 2 Adenocarcinomas of the distal colon at the 21st week of DMH treatment in group C. Magnification is × 400.

significantly at the 9th week. The enzyme activity did not increase statistically until the 11th week in group B. After the 16th week of the experimental period, the activity of MnSOD in all experimental groups returned to the level of the control group. The data indicated that DMH could also induce an increase of MnSOD activity in the early stages of carcinogenesis and cholic acid could increase the MnSOD activity induced by DMH treatment two weeks earlier. Indole, on the contrary, delayed the increase of MnSOD activity two weeks.

Figures 5 and 6 show the temporal changes of SOD activities during DMH large intestine carcinogenesis compared with the control group. In animals given DMH

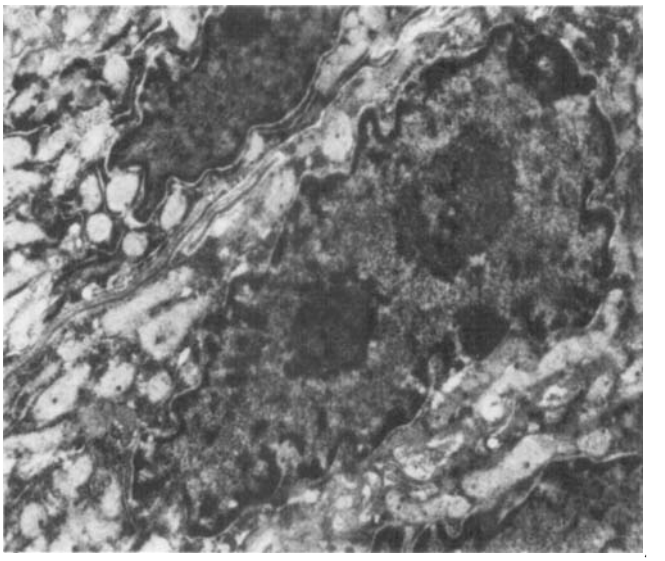

FIGURE 3 Adenocarcinoma cell of the distal colon at the 21st week of DMH treatment in group C. Magnification is × 10,400.

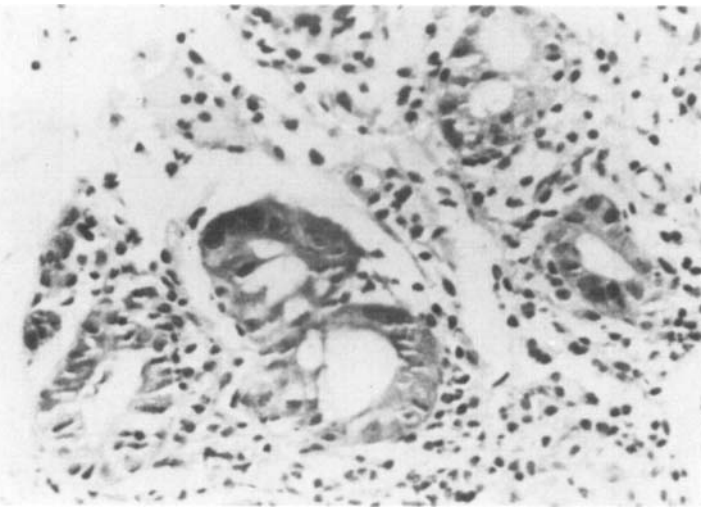

FIGURE 4 Mucosa of the distal colon at the 21st week of DMH treatment in group A. Severely deranged mucosa with focal atypias are seen. Magnification is × 400.

alone (group D), Cu,ZnSOD did not increase until 16th week of experiment when the anaplasia grade I of colon mucosa appears; the enzyme activity remained at an increased level at 21 weeks (anaplasia grade II) and reached the highest level when cancer formed (See Table II). On the other hand, the increase of MnSOD activity took

TABLE I
Temporal changes of SOD activities of mouse large intestinal mucosa during DMH carcinogenesis[a,b]

Experimental week	SOD	Group A DMH + C + I	Group B DMH + I	Group C DMH + C	Group D DMH	Group E Control
Seventh	Cu-ZnSOD	39 ± 3*(5)	26 ± 1(5)	24 ± 3(5)	28 ± 1(5)	30 ± 2(5)
	MnSOD	10 ± 1	9 ± 1	16 ± 1**	7 ± 1	8 ± 1
Ninth	Cu-ZnSOD	34 ± 2**(6)	30 ± 3(6)	29 ± 2(6)	26 ± 3(6)	21 ± 2(6)
	MnSOD	17 ± 2**	11 ± 2	15 ± 2*	14 ± 1*	8 ± 1
Eleventh	Cu-ZnSOD	36 ± 2**(6)	22 ± 1(6)	28 ± 2(5)	27 ± 3(6)	23 ± 2(6)
	MnSOD	21 ± 1**	19 ± 1**	20 ± 1**	19 ± 1**	12 ± 1
Sixteenth	Cu-ZnSOD	39 ± 2*(6)	41 ± 2*(6)	40 ± 3*(5)	39 ± 2*(5)	30 ± 2(6)
	MnSOD	19 ± 2	20 ± 1	16 ± 1	15 ± 1	16 ± 1
Twenty-first	Cu-ZnSOD	43 ± 2*(7)	44 ± 3*(6)	42 ± 2*(4)	40 ± 3*(6)	31 ± 2(6)
	MnSOD	19 ± 1	19 ± 1	18 ± 1	17 ± 1	17 ± 1

[a] Values are mean ± SEM. All errors represent 1 SEM. Numbers in parentheses are numbers of animals. Statistical analysis was made by Analysis of Variance.
* 95% significance, ** 99% significance compared with control group.
[b] Enzymatic units: U/mg protein. One unit of SOD was defined as the amount of protein that resulted in 50% inhibition of the rate of NBT reduction.

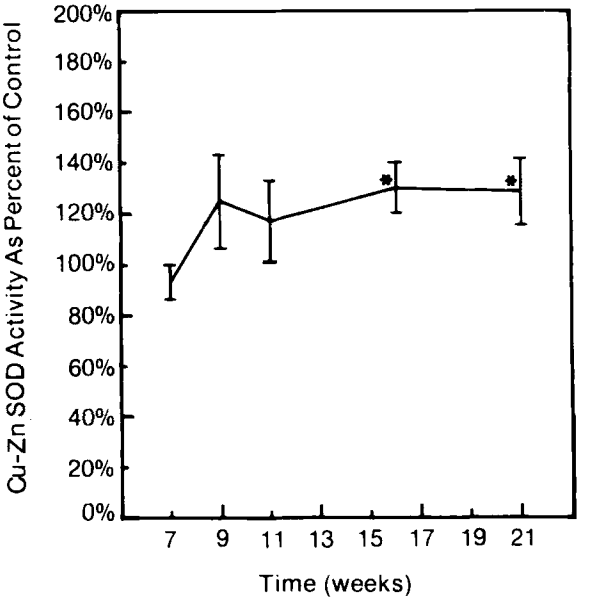

FIGURE 5 Temporal changes in Cu-ZnSOD activity during DMH large intestinal carcinogenesis. Graph represents per cent of control group's Cu-ZnSOD activity, with the control group arbitrarily set to 100%. Statistical difference at the 95% level between control (group E) and treatment (group D) groups are indicated by asterisks.

place at the 9th week when no morphological changes occurred, but decreased to normal level at the 16th week when the anaplasia grade I appeared and decreased significantly at the time of cancer formation (See table II).

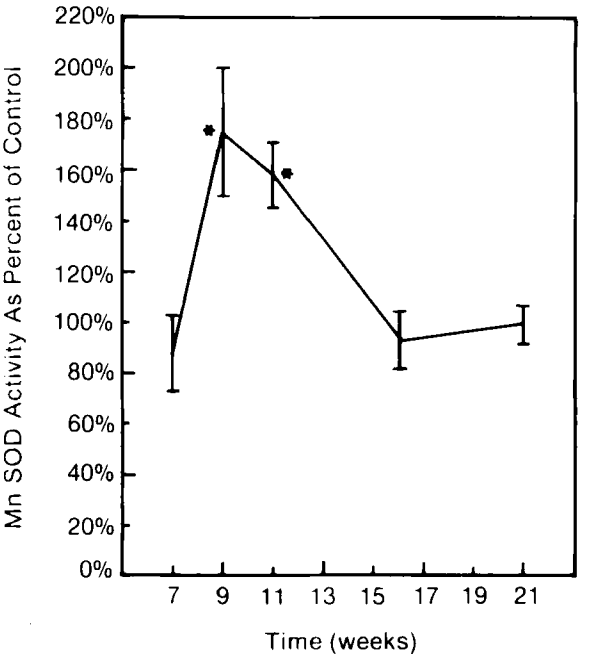

FIGURE 6 Temporal changes in MnSOD activity during DMH large intestinal carcinogenesis. Graph represents per cent of control group's MnSOD activity, with the control group arbitrarily set to 100%. Statistical difference at the 95% level between control and treatment groups are indicated by asterisks.

Table II shows the SOD activities of intestinal cancer tissues. Mouse colon cancer was induced by DMH injection weekly for one year with a dose of 20 mg/kg bw. The results indicated that colon cancer had a higher Cu,ZnSOD activity than the mucosa of colon around the tumor and than completely normal mucosa of 27 week old mice. The large intestinal mucosa around the tumor had statistically different MnSOD activity from the completely normal mucosa. Loven *et al.* has also reported this phenomena.[5] The Cu,ZnSOD but not MnSOD activity in the tumor was different statistically at the 0.05 level from the large intestinal mucosa surrounding the tumor;

TABLE II
The SOD activity of mouse large intestine cancer induced by DMH[a,b]

SOD	Cancer Tissues	Large Intestinal Mucosa Around Cancer	Normal Large Intestinal[c] Mucosa at 27 Weeks of Age
Cu-ZnSOD	63 ± 2**(7)	42 ± 2*(7)	31 ± 2(6)
MnSOD	10 ± 1**(7)	12 ± 1*(7)	17 ± 1(6)

[a] Values are means ± SEM. All errors represent 1 SEM. Numbers in parentheses are numbers of animals.

* 95% significance, ** 99% significance compared with normal large intestinal mucosa from mice at 27 weeks of age.

[b] Enzymatic units: U/mg protein. One unit of SOD was defined as the amount of protein that resulted in 50% inhibition of the rate of NBT reduction.

[c] These control mice were the same as used in Table I at 21 weeks.

however, both of the enzymes in the tumor were different at the 0.01 level from the completely normal mucosa.

DISCUSSION

Several investigations have reported that MnSOD activity decreased as the pH increased. These results were from 1) pulse-radiolysis study of enzyme in which MnSOD was 6-fold decreased.[22] 2) studies using xanthine oxidase/cytochrome c which showed about 5-fold decrease of SOD activity[23] or not very strongly affected by pH changes.[24] 3) direct assay which showed MnSOD had about 2-fold decrease as the pH increased from 8.9 to 10.2.[25] However, Crapo *et al.* reported that with the xanthine oxidase/cytochrome c system, the activity of the Cu,ZnSOD appears to be approximately 10 times greater at pH 10.0 than at pH 7.8, whereas the MnSOD appears only about twice as active at the high pH.[26] Using our xanthine oxidase/NBT system, we found that as the pH increased from 7.8 to 10.2, the sensitivity for bovine liver Cu,ZnSOD was approximately 10-fold increased. However, MnSOD purified from chicken liver showed a 5-fold decrease (unpublished observations). It could be concluded that both pH and assay method would influence the activity of MnSOD. Our assay does detect reasonable levels of MnSOD and it appears appropriate for this study.

It is well known that all aerobic living beings can produce superoxide radicals (O_2^-). Some carcinogens such as benzo(a)pyrene, hydrazine and N-nitroso compounds and promoters, such as phorbol esters, can increase the formation of O_2^-.[12] Superoxide radicals have been shown to peroxidize lipids, damage cell membranes, depolymerize polysaccharides, cleave DNA, cause chromosomal aberrations, mutation, carcinogenesis and kill cells.[27–29] SOD (EC 1.15.1.1), which catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen, can protect the biological macromolecules against oxidation and decomposition,[30,31] prevent chromosome breakage,[32] inhibit cellular transformation[33] and tumor promotion.[28] Our work showed that Cu,ZnSOD and MnSOD of intestinal mucosa increased at 16th and 9th week of the experimental period, respectively, during DMH-induced carcinogenesis. MnSOD activity returned to control levels after the 16th week. The additive action of indole and cholic acid on DMH caused the increase of Cu,ZnSOD activity to appear at least four weeks earlier. Cholic acid also caused the increase of MnSOD activity to occur earlier. However, indole showed an inhibitory effect on the elevation of MnSOD activity induced by DMH injection. The elevation of SOD activity during DMH carcinogenesis seems to be related to superoxide radical produced by DMH. It has been reported that the changes of free radical content were found in frozen tissue from various type of cancers induced by viruses, chemicals or hormones. In most cases, a decrease in the free radical concentration was observed after the first several weeks following administration of the carcinogens. Later the free radical content markedly increased and attaining a maximum value in proliferating pretumor foci and primary tumor nodes. However, after further tumor growth, the radical content fell below normal values.[34] The change of the free radical concentration is somewhat correlative with the change of MnSOD activity during carcinogenesis. At the early stage of DMH carcinogenesis the increase of MnSOD activity may result from the induction of superoxide radical produced by DMH. However, when the tumor was formed, the radical content might fall below normal values at the time

when the MnSOD activity decreased significantly. The increase in MnSOD activity seen during DMH carcinogensis does not seem consistent with the fact that the tumors themselves have lowered MnSOD activity. The lowering of MnSOD and increase in Cu,ZnSOD activity in large intestinal tumors compared to an appropriate control has also been seen by Loven *et al.*[5] In fact, the percentage changes seen in both forms of the enzyme are similar in both studies. Thus, the changes appear to be reproducible. The most logical explanation why MnSOD is not lowered in the early stages of DMH carcinogenesis is that only a few cells are malignant at these early stages; thus, any changes in SOD will be masked by the normal cell population. As clonal expression and cell proliferation occur, the percentage of transformed cells increase dramatically and the SOD changes can be seen.

In addition we found that MnSOD activity of normal mouse large intestinal mucosa increased as the experiment progressed. Increases in SOD activity during developmental aging are a common finding. Oberley and Oberley[35] have reviewed the studies shown that MnSOD is associated with cell differentiation. The activity of this enzyme has been shown to greatly increase during the development of the fruit fly and differentiation of advanced organisms. The reason for this association might be that differentiation of some cells is associated with an increase in mitochondrial respiration and the concomitant high ATP formation. More mitochondrial respiration will mean more oxygen free radical production and hence an increase in MnSOD is necessary.

Cohen *et al.* reported that 0.2% cholic acid feeding could increase the concentration of total bile acid, especially sodium deoxycholate, in the gut.[17] Using a high performance liquid chromatography method, we found that the contents of the large intestine of mice fed with 0.8% indole contained a 10-fold increased indole level (unpublished data). Bile acids have been regarded as promoters of large intestinal cancer. The mechanisms involved may include 1) increased intestinal permeability and absorption of dimethylbenz(a)anthracene and dimethylhydrazine[36] 2) increased DNA replication in rat colon[37] 3) breakage of DNA chains and promotion of unscheduled DNA synthesis *in vitro*[38] 4) induction of ornithine decarboxylase activity in mouse colon mucosa[39] 5) stimulation of colon epithelial cell proliferation and enhancement of the expression of malignantly transformed cells[17] 6) induced increases in reactive oxygen species which are linked to the subsequent stimulation of the proliferation of colonic epithelium.[14] Our experiment indicated that cholic acid could promote the formation of large intestinal cancer induced by DMH. The additive action in the induction of Cu,ZnSOD and MnSOD activities of large intestinal mucosa during DMH carcinogenesis might be another mechanism of action of bile acids.

Hill once classified indole as one of promoters of intestinal cancer.[40] However, few studies have been performed on the relationship between large intestinal cancer and indole. Our present experiment showed that indole was observed to have an inhibitory effect on the induction of MnSOD by DMH. Furthermore, the morphological observations indicated that indole might decrease the action of cholic acid as a promoter of large intestinal cancer although it had no effect by itself on DMH large intestine carcinogenesis.

In conclusion, our results indicated that DMH, as a free radical producer, could induce Cu,ZnSOD and MnSOD activities during carcinogenesis and cholic acid and indole might have some promoting and inhibitory effects on this induction. The experiment may imply one mechanism of action of DMH as a carcinogen and cholic acid as a promoter in large intestinal cancer.

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