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Protective Roles of Mushrooms in Experimental Colon Carcinogenesis

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There is epidemiological evidence that the population with high fecal β-glucuronidase activity has greater risk of colon cancer than the population with low fecal β-glucuronidase. This relationship was investigated by using the mouse-dimethylhydrazine colon carcinogenesis model and the fraction of G.lucidum which is a β-glucuronidase inhibitor. Mice with low fecal β-glucuronidase activity induced by consumption of the ether fraction of G.lucidum had significantly fewer aberrant crypts(AC) after injections of 1,2-dimethylhydrazine (DMH) than mice treated with DMH alone. The result supports the hypothesis that the inhibitor such as the ether fraction of G.lucidum can protect an animal against the induction of colon cancer.

Key words : β-glucuronidase, Mushroons, Colorectal cancer, Aberrant crypts

INTRODUCTION

Epidemiological studies suggest that the dietary factors, such as high animal fat and protein, are prime factors in the etiology of colon cancer (Goldin and Gorbach, 1976: Weisburger, 1977). The following hypothesis has been suggested by many researchers (Hill, 1975: Reddy *et al.*, 1975: Wynder and Reddy,1974). Dietary fat changes bile acid and cholesterol metabolites quantitatively and qualitatively, as well as the concentration and metabolic activity of bacteria in the colon, which may produce carcinogen or carcinogenic compounds from bile acid and cholesterol metabolites. Intestinal bacteria may play an important role in liberating active key intermediates with chemical carcinogens inducing colon tumors in experimental animals (Goldin and Gorbach, 1976: Weisburger, 1971). We are currently engaged in the study of bacterial enzymes, especially β-glucuronid-ase, and the metabolism of DMH. It is thought that DMH injected into the rat may be conjugated with glucuronic acid immediately in the liver and secreted via the bile to the intestine. The glucuronic acid conjugate would be hydrolyzed by bacterial β-glucuronidase to the free compound, producing a relatively high localized concentrat-ion of this compound in the colonic mucosa. This active carcinogen causes colonic cancer (Fiala, 1975 and 1977). If fecal β-glucuronidase could be inhibited, the postulated glucuronic acid-conjugated carcinoma by DMH should be prevented. Therefore, we extended our study to elucidate the effect of intestinal bacterial β-glucuronidase on the carcinogenicity of DMH in the colon, using mushrooms as the β-glucuronidase inhibitor.

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MATERIALS AND METHODS

Chemicals

1,2-Dimethylhydrazine (DMH), p-nitrophenylsulfate, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-glucuronide and methylene bule were purchased from Sigma Chem. Co. (U.S.A.). Brain heart infusion broth was from Difco Co. (U.S.A.)

Extraction and fractionation

Mushrooms(100 g) were extracted with 70%-methanol (500 ml, 3 times) at 80°C. The 70% methanol extract was concentrated. In order to fractionate the extract of G. lucidum, 1kg of G. lucidum was extracted with 5L of 70% methanol. This extract was fractionated successively with diethylether (14.8 g), ethylacetate (2.1 g) and then n-buthanol (1.2 g). The residual fraction (11.6 g) also was concentrated.

Animal

In all studies the animals were housed in plastic

cages with wire tops. Unless otherwise stated, the animals were fed Samyang ad libitum and had free access to water. The mice (ICR male 15 g) were divided to 6 groups. Three groups (Group II, III, IV) were given weekly s. c. injection of DMH (20 mg per kg of body weight per week) for 10 weeks. The other groups (Group I, V, VI) were given weekly s.c. injections of saline not containing DMH. Group I and Group II were fed a usual powdered laboratory diet. Group III was fed a powdered diet containing a 70% methanol extract of G. lucidum (1.5 mg/mouse/day). Group IV was fed a powdered diet containing the diethylether extract of G. lucidum (0.75 mg/mouse/day). Grorp V was fed a powdered diet containing the 70% methanol extract of G. lucidum (1.5 mg/mouse/day). Group VI was fed a powdered diet containing the diethylether extract of G. lucidum (0.75 mg/mouse/day). After the last injection, all animals were fed 5 weeks and then autopsied.

Visualization and quantification of aberrant crypts (AC)

After termination of the animal, the colon was removed immediately, flushed with Kreb's Ringer, slit open from caecum to anus, and fixed in 10% buffered formalin. Following the previously cited protocol (McLellan and Bird, 1988: Rao*at al.*, 1993) the fixed colons were stained with methylene blue and the colons were assessed for AC by using the light microscope. The parameters used to assess the colons were occurrence, (size), and distribution of AC. The occurrence was measured by quantitating the mean number of foci of AC per colon. The number of AC per focus was also recorded. To determine the distribution of AC, the colon was divided into two sections. Rectum represented the first 2cm from the rectal end. Sigmoidal and desending colons were the next 2.5cm respectively from the rectum.

Preparation and assay of enzyme activity (Kim and Kobashi 1986: Kim *et al.* 1994)

All preparatory procedures for the enzyme determination were carried at 0-4°C. All determinations were performed in duplicate on each sample. All enzyme activities were measured weekly in each group. For the assay of β-glucosidase, the reaction mixture consisting of 0.4 ml of 2 mM p-nitrophenyl-β-D-glucopyranoside, 0.6 ml of 0.1 M phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution was prepared. For the assay of β-glucuronidase, the reaction mixture consisting of 40 μl of 10 mM p-nitrophenyl-β-D-glucuronide, 0.76 ml of 0.1 M phosphate buffer, pH7.0, and 0.2 ml of enzyme solution was prepared. For the assay of arylsulfate sulfotransferase, the reaction mixture consisting of 60 μl of 50 mM p-nitrophenyl sulfate, 0.58 ml of 0.1 M phosphate buffer, pH 8, and 0.2 ml of enzyme solution was prepared. Each reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 0.25 N NaOH. The reaction mixture was centrifuged at 1000xg for 20 min. Then, the absorbance of the reaction mixture was measured at 405 nm within 10 min.

Partial purification of β-glucuronidase

HGU-3 β-glucuronidase was purified as follows: HGU-3, alkalotolerant human intestinal bacterium was cultured in a 10 L BHI broth and collected at 3000×g for 30 min at 4°C. The precipitate was suspended with 50 mM phosphate buffer, pH 7, and sonicated. The resulting supernatant was fractionated with ammonium sulfate and then applied to DEAE-cellulose column chromatography and saccharic acid 1, 4-lactone-legand-affinity column chromatography. The specific activity of the purified enzyme was 0.125 μmol/ml/mg protein. Lysosomal β-glucuronidase was partially purified from rat liver according to Brand and Hess (1983) and the specific activity was 0.132 μmol/min/mg protein.

RESULTS

Inhibition of β-glucuronidase(s) by the methanol extract of mushrooms.

The inhibition against β-glucuronidase of HGU-3, which is human intestinal bacterium, and that of rat lysosome by 70% methanol extractable fractions of mushrooms is shown in Table 1. In the HGU-3 β-glucuronidase, the G. lucidum extract showed the best inhibition of the enzyme, followed by T. caligatum and L. edodes. In the rat lysosomal β-glucuronidase, L. edodes was the best inhibitor. However, the other

Table 1. Inhibitory power of the mushrooms extract on β-glucuronidase

Mushroom	Inhibition (%)	
	HGU-3**	Lysosome***
Lentinus edodes	32.5	44.4
Ganoderma lucidum	84.7	28.1
Coriolus versicolor	32.4	1.7
Pleurotus ostreatus	-0.2	10.5
Tricholoma caligatum	35.6	7.9
Auricularia auricula	31.4	4.7
Gyrophora esculenta	31.4	8.8
None	0.0	0.0

*Final concentration was 2 mg/ml.
**Activity (0.125 μmole/min/mg protein) was taken as 100%
***Activity (0.132 μmol/min/mg protein) was taked as 100%

Table II. Inhibitory potency of each fraction of G. lucidum on β-glucuronidase

Fraction	IC_{50} (mg/ml)	
	Rat Microsome*	HGU-3**
ethyl acetate Fr.	0.47	0.52
buthanol Fr.	1.49	1.40
water Fr.	2.50	5.30
none	0.00	0.00

*Activity (0.133 μmole/min/mg protein) was taken as 100%.
**Activity (0.125 μmole/min/mg protein) was taken as 100%.

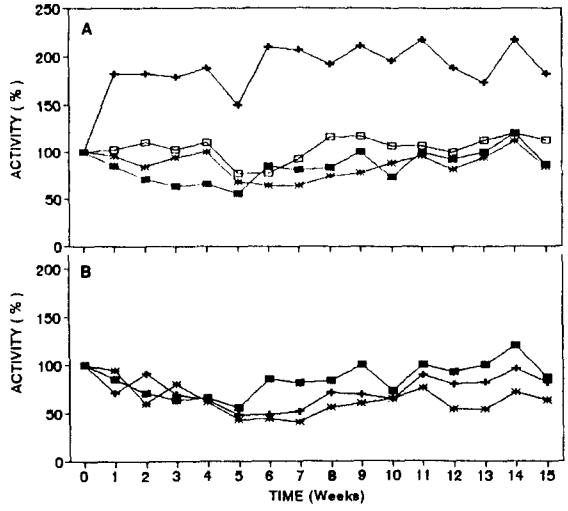

Fig. 1. Change of β-gluruonidase activity of intestinal bacteria by the administration of DMH (s.c.) and/or G. lucidum (p.o.). A: Group 1, ■; Group 2, +; Group 3, *; Group 4, □. B: Group 1, ■; Group 5, +; Group 6, *. Activity (0.38 μmole/min/g wet feces) was taken as 100%.

methanol-extractable fraction of these mushrooms were weak inhibitors

Inhibition of each fraction of G. lucidum on β-glucuronidase(s)

The 70% methanol extract of G. lucidum, which was the best inhibitor on HGU-3 β-glucuronidase, was fractionated stepwise with diethylether, ethylacetate and n-butanol. Inhibition of each fraction of G. lucidum on β-glucuronidase of HGU-3 and rat lysosome was investigated (Table II). The ether fraction was the best inhibitor of β-glucuronidase of HGU-3 and rat lysosome, followed by ethylacetate fraction, butanol fraction and the residual fraction.

Effect of the ether fraction of G. lucidum on fecal enzymes activities.

The effect of G. lucidum, which inhibited bacterial β-glucuronidase in vitro, was investigated in vivo by using the mouse-dimethylhydrazine colon carcinogenesis model (Fig 1). By injection of DMH to the mouse weekly for ten weeks, fecal β-glucuronidase activity was induced 1.5-2.2-fold. However, β-glucosidase and arylsulfate sulfotransferase were not induced (data not shown). The fecal β-glucuronidase activity was induced after 2 weeks injections of 1,2-dimethylhydrazine, and persisted throughout the experiment. This induction seems to be slightly related to fecal pH, because fecal pH was also increased af ter 2 weeks DMH injection. However, by treating G. lucidum, the β-glucuronidase activity was decreased 55-96%, compared to control. On the case of mice untreated with DMH, the enzyme activity was decreased similarly by administering G. lucidum. The enzyme was inhibited more effectively in vivo by the ether fraction than 70% methanol extract. This result coincided with the in vitro results.

Table III. Effect of the ether fraction of G. lucidum on formation of AC in mouse colon induced by DMH

Group	Incidence	NO.ACF/colon*	NO.AC/focus	Distribution	
				R	S & D
1	0/9	0	0	0	0
2	8/9	73.7	16.2	89	11
3	7/9	45.4	10.8	85	15
4	3/9	23.0	9.2	100	0
5	0/9	0	0	0	0
6	0/9	0	0	0	0

*Number of aberrant crypt foci per colon

Effect of the ether fraction of G. lucidum on colon carcinoma

Whether the population with high fecal β-glucuronidase activity had greater risk of colon cancer than the population with low fecal β-glucuronidase or not was investigated by using the mouse-dimethylhydrazine colon carcinogenesis model and the ether fraction of G. lucidum, which was a β-glucuronidase inhibitor in vitro (Table III, Fig 2). The incidence of AC in the 6 groups of rats was as follows: Group 1, 0%: Group 2, 89%: Group 3, 78%: Group 4, 33%: Group 5, 0% and Group 6, 0%. The average number of ACF was 0, 73.7, 45.4, 23.0, 0, 0 AC/colon. In Group 2, two mice died within the 8 to 11 week period. Particularly, mice with low fecal β-glucuronidase activity, produced by consumption of the ether fraction or 70% methanol extract of G. lucidum, had significantly fewer AC after injections of DMH than mice treated with DMH alone. The inhibitory effect of the ether fraction of G. lucidum was higher than that of 70% methanol extract. The inhibitory mode was similar to that of β-glucuronidase in vitro. The other groups were not affected by G. lucidum.

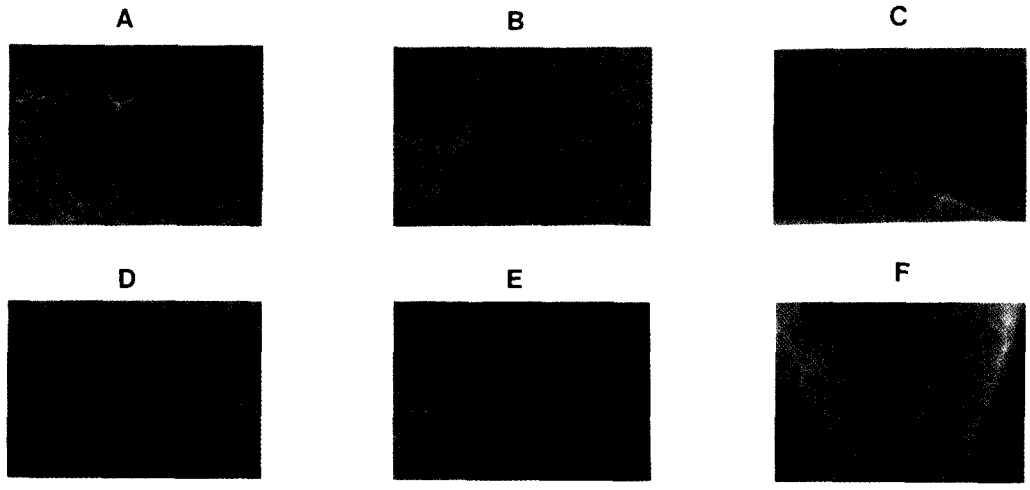

Fig. 2. Topological views of AC in unsectioned methylene blue-stained murine colon of Group 1 (A), Group 2 (B) Group 3 (C), Group 4 (D), Group (E) and Group 6 (F).

DISCUSSION

There is epidemiological evidence that the popualtion with high fecal β-glucuronidase activity had a greater risk of colon cancer than the population with low fecal β-glucuronidase. This relationship was investigated in the laboratory by using the mouse-dimethylhydrazine colon carcinogenesis model and the ether fraction of G. lucidum as a β-glucuronidase inhibitor. The mouse colon carcinoma model was made by injection of DMH according to Reddy et al. (1975). By using this model, ACs on the colon of mice were produced well and bacterial β-glucuronidase was induced about 2-fold. Therefore, formation of ACs was related to bacterial β-glucuronidase. This result supported epidemiological evidence of colon cancer. The ether fraction of G. lucidum was used because the G. lucidum extract was the best inhibitor among mushrooms in vitro. Mice with low fecal β-glucuronidase activity, produced by consumption of the ether fraction of G. lucidum had significantly fewer AC after injections of DMH than mice treated with DMH alone. The other enzymes, β-glucosidase and arylsulfate sulfotransferase, were not related to colon cancer. In addition, the average number of AC, size of AC and mortality were improved by treatment with G. lucidum which was a potent inhibitor of β-glucuronidase. The inhibitory effect of the ether fraction of G. lucidum was more potent than that of its 70% methanol extract. The inhibitory model was similar to that of in vitro β-glucuronidase. It can be suggested that the ether fraction of G. lucidum reduce the risk factor of colon cancer as well as liver damage (Kim *et al.* 1994) by inhibiting the hydrolysis to glucuronides of proximate metabolism.

This result supports the hypothesis that the population with high β-glucuronidase activities had a greater risk of colon cancer than those with low β-glucuronidase and its inhibitors can protect against the induction of colon cancer.

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