[CANCER RESEARCH 42, 331-334, January 1982]
0008-5472/82/0042-0000$02.00

Effect of β-Glucuronidase Inhibitor on Azoxymethane-induced Colonic Carcinogenesis in Rats[1]

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ABSTRACT

In this study of the role of microfloral β-glucuronidase in colonic carcinogenesis, the effect of β-glucuronidase inhibitor was evaluated. Starting at 5 weeks of age, male Donryu rats were fed either a semisynthetic diet or the same diet containing 0.1% β-glucuronidase inhibitor as *N*-cyclohexyl-5-*O*-acetyl-2,4-*O*-(*p*-methoxybenzylidene)-D-glucaro-1-amide-6,3-lactone (C-GAL). All animals were given s.c. injections of 7.4 mg azoxymethane (AOM) per kg body weight once a week for 11 weeks and followed for an additional 20 weeks. Most animals receiving the colonic carcinogen developed tumors in the colon, and a few also developed tumors in the small intestine. However, the number of tumors in the large intestine of the rats given C-GAL at the same time as AOM was significantly lower than in the control rats, especially in the proximal half of the colon, but those given C-GAL after AOM treatment had almost the same number of colon tumors as did the controls. It is concluded that, since bacterial β-glucuronidase activity in the feces of rats given 0.1% C-GAL was significantly inhibited, intestinal microfloral β-glucuronidase may play an important role in colonic carcinogenesis caused by AOM.

INTRODUCTION

Epidemiological studies suggest that dietary factors, particularly high animal fat and protein, are prime factors in the etiology of colon cancer (6, 11, 20, 45). The following hypothesis has been suggested (22, 24, 39, 42, 46). 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 (8, 32, 43). We are currently engaged in studies of bacterial enzymes, especially β-glucuronidase, and of the metabolism and mode of action of AOM.[3] It is thought that AOM injected into the rat is hydroxylated by the microsomal monooxygenase system in the liver (17) and may be conjugated with glucuronic acid immediately upon formation and transported via the bile to the intestine (29, 43). This glucuronic acid conjugate, which is presumably stable, would then be hydrolyzed by bacterial β-glucuronidase to free MAM, producing a relatively high localized concentration of this compound in the colonic mucosa. MAM, an active carcinogen, readily and spontaneously yields the alkylating methyl carbonium ion, capable of reacting with cellular and molecular targets plus other products (2, 3, 8, 15, 16, 23, 32, 41, 43). If fecal β-glucuronidase could be inhibited, the postulated glucuronic acid-conjugated carcinogen would be excreted in the feces, and the induction of colon carcinoma by AOM should be prevented.

Therefore, we extended our studies to elucidate the effect of intestinal microfloral β-glucuronidase on the carcinogenicity of AOM in the colon, using a β-glucuronidase inhibitor. Several D-glucaric acid derivatives were tested, and C-GAL, potent inhibitor of fecal β-glucuronidase, was chosen for the present study (Chart 1).

MATERIALS AND METHODS

Chemicals. AOM, purchased from Ash Stevens Co., Detroit, Mich., was dissolved in 0.9% NaCl solution. C-GAL was generously provided by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. Other biochemicals were products of Sigma Chemical Co., St. Louis, Mo.

Animals. Weanling male Donryu rats were obtained from Kitayama Labs, Inc., Kyoto, Japan, and kept in a temperature- and humidity-controlled clean room. The animals were divided into 4 groups as in Chart 2, fed the usual powdered laboratory diet (CE-II; CLEA Japan, Inc., Tokyo, Japan) or the same diet containing 0.1% C-GAL, and given water *ad libitum*. Starting at 6 weeks of age, the rats were given 11 weekly s.c. injections of AOM (7.4 mg per kg of body weight per week). All animals were autopsied 20 weeks after the last injection.

All organs including the intestine were examined grossly and histologically for number and type of tumors, and the size and location of intestinal tumors were recorded. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and the sections were stained with hematoxylin and eosin.

Preparation and Assay of Enzyme Activity. All preparatory procedures for enzyme determinations were carried out at 0-4°. All determinations were performed in duplicate on each sample. Fecal β-glucuronidase activity was assayed 3 times in the course of this study: 3 weeks after the first injection of AOM; at the completion of AOM treatment; and again at the time of sacrifice. The colonic contents were diluted with ice-cold 0.9% NaCl solution and centrifuged at 15,000 × *g* for 30 min at 4° in an ultracentrifuge. The pellet containing bacteria was suspended in ice-cold distilled water and sonicated for 7 min at 0°. This material was filtered through a stainless steel filter. β-Glucuronidase activity was assayed as described by Fishman (18). The reaction mixture, containing 0.1 ml 0.01 M phenolphthalein glucuronide at pH 5.5 with 0.8 ml 0.1 M acetate buffer and 0.1 M of enzyme source, was incubated for 30 min at 37° in a water bath. The reaction was terminated by adding 2.5 ml of 0.1 M glycine solution and 1 ml of 5% trichloroacetic acid. The phenolphthalein liberated was measured at 540 nm in a Hitachi spectrophotometer. The β-glucuronidase activity was expressed as μg phenolphthalein liberated per mg wet weight of colonic content per hr at 37°. The results are expressed as mean ± S.D.

Statistical Analysis. The data were analyzed with Student's *t* test, and the levels of significance were expressed as *p* values.

[1] Supported by a Grant-in-Aid for Scientific Research of the Ministry of Education, Science, and Culture of Japan.

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[3] The abbreviations used are: AOM, azoxymethane; MAM, methylazoxy methanol; C-GAL, *N*-cyclohexyl-5-*O*-acetyl-2,4-*O*-(*p*-methoxybenzylidene)-D-glucaro-1-amide-6,3-lactone; DMH, dimethylhydrazine; i.r., intrarectal.

Received August 30, 1979; accepted September 6, 1981.

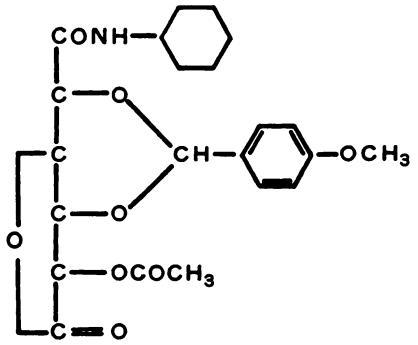

Chart 1. Structure of C-GAL.

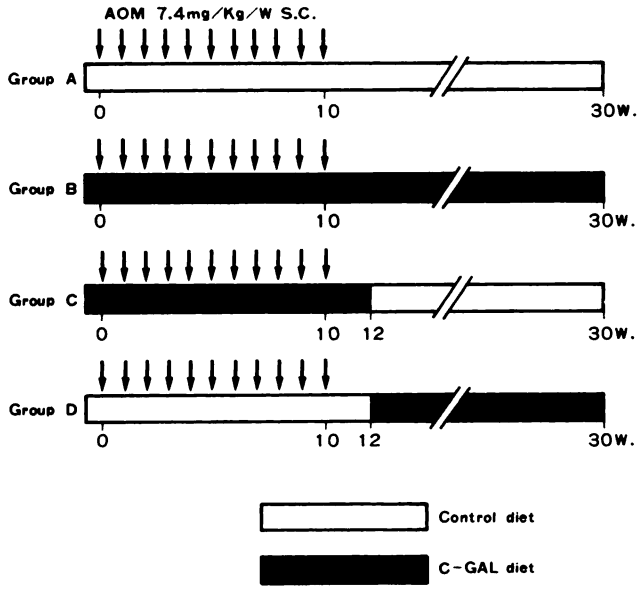

Chart 2. Male Donryu rats were divided into 4 groups and fed either a usual laboratory diet (*control diet*) or the same diet containing 0.1% C-GAL (*C-GAL diet*). In Groups C and D, the diet was changed 2 weeks after the last injection of AOM. All animals were given weekly s.c. injections of AOM 11 times and were autopsied 20 weeks after the last injection. *W*, week(s).

RESULTS

β-Glucuronidase Activity of Colonic Contents. Table 1 summarizes the activity of β-glucuronidase in the contents of the proximal and the distal halves of the colons of rats fed the control or the C-GAL diet at the 3 periods of this experiment: 3 weeks after the first injection of AOM; at the completion of AOM treatment; and at the time of sacrifice. The fecal β-glucuronidase activity of rats fed the C-GAL diet was significantly inhibited in both the proximal and distal halves of the colon 3 weeks after the first injection of AOM and at the completion of AOM treatment, but not at the time of sacrifice, when the colonic contents were bloody, fermented, and muddy because of tumor or intussusception. The rats were all weighed weekly. The mean weight gain in all 4 groups was almost the same with no particular effect of C-GAL.

Tumor Induction. Table 2 summarizes the incidence of tumors in the 4 groups of rats treated with AOM: Group A, 100%; Group B, 97%; Group C, 92%, and Group D, 100%. The average number of colon tumors per rat was 9.9 ± 6.3 in Group A (control group), 6.4 ± 4.5 in Group B, 3.1 ± 4.0 in Group C, and 11.1 ± 7.9 in Group D. Thus, the average number of colon tumors was significantly suppressed in Group B ($p < 0.05$) and Group C ($p < 0.001$). The distribution of these neoplasms in the proximal and distal halves of the colon is shown in Table 2. Tumors occurred throughout the colon in Groups A and D, but the number of tumors was significantly reduced in the proximal half in Groups B ($p < 0.001$) and C ($p < 0.001$). Furthermore, in Group C, the average number of tumors in the distal half was significantly reduced ($p < 0.02$). About 60% of the tumors were polypoid, and polypoid or intramural tumors often caused intestinal obstruction, bloody stools, and intussusception. The diameter of the colon tumors varied from 0.1 to 3.1 cm, and the distribution pattern according to size was almost the same in each group. Carcinomatosis of the peritoneum was present in a few rats, but liver and lung metastases were not found. No tumors were found in other organs, and no tumors were found in the intestine or other organs of rats not given AOM.

Histology. The majority of the colonic tumors were well-differentiated tubular adenocarcinomas, and in a few cases, signet-ring cell carcinomas were found invading the lamina propria or the serosa. There were no histological abnormalities in the liver, kidney, or spleen of rats fed the C-GAL diet.

DISCUSSION

Several studies have indicated that dietary factors are most important in the etiology of colon cancer (9, 11, 12, 40, 47). The nature of the diet affects the composition of the intestinal bacterial flora, and bacteria can produce carcinogen or cocar-

Table 1

β-Glucuronidase activity of colonic contents of rats treated with AOM

	Proximal half	Distal half
Control diet group		
I[a]	9.05 ± 1.77[b, c]	12.83 ± 1.65
II[d]	12.05 ± 5.81	12.12 ± 4.49
III[e]	6.94 ± 4.03	8.14 ± 4.42
C-GAL diet group		
I[a]	4.43 ± 3.07[f]	4.55 ± 2.75[g]
II[d]	4.51 ± 1.48[f]	5.37 ± 3.85[f]
III[e]	10.40 ± 9.68	5.27 ± 3.01

[a] Three weeks after the first injection of AOM.
[b] μg phenolphthalein per mg, wet weight, of colonic content per hr.
[c] Means ± S.D.
[d] After 11 weekly injections of AOM.
[e] At sacrifice (20 weeks after last injection).
[f] Significantly different from control diet group at each period ($p < 0.05$).
[g] $p < 0.01$.

Table 2

Incidence of colon carcinoma in rats treated with AOM

Starting at 6 weeks of age, the rats were given 11 weekly s.c. injections of AOM, 7.4 mg/kg body weight. All animals were autopsied 20 weeks after the last injection.

	Rat		No. of colon tumor/rat		
	No.	% with tumor	Total	Proximal half	Distal half
Group A	19	100	9.9 ± 6.3[a]	4.8 ± 3.5	5.1 ± 3.7
Group B	32	97	6.4 ± 4.5[b]	1.0 ± 1.3[c]	5.5 ± 4.2
Group C	25	92	3.1 ± 4.0[c]	0.8 ± 1.1[c]	2.2 ± 3.6[d]
Group D	14	100	11.1 ± 7.9	4.5 ± 4.3	6.6 ± 5.3

[a] Mean ± S.D.
[b] Significantly different from Control Group A ($p < 0.05$).
[c] $p < 0.001$.
[d] $p < 0.02$.

cinogen from dietary components or from endogenous secretions produced in response to the diet (1, 4, 25, 26, 33, 48). A high animal protein and/or animal fat diet, similar in composition to that consumed by humans with a relatively high risk of colon cancer, is associated with elevated levels of β-glucuronidase in the colonic contents in both humans (46) and rats (20, 35, 38) but not in the colonic mucosa (35). Furthermore, elevated levels of nitroreductase and azoreductase in the colonic microflora have been found to be associated with a high-beef diet (20). These enzymes are implicated in the conversion of procarcinogen to carcinogens.

Experimental animal models have been developed to study colon carcinogenesis by DMH (5, 13, 21, 36, 37) and AOM (10, 14, 19, 36). Reddy *et al.* (37) noted that fewer tumors were found in germ-free than in conventional rats treated with DMH, which needs metabolic activation, but i.r. injection of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a direct-acting carcinogen, nearly doubled the number of tumors. Reddy *et al.* (36) also investigated the effect of intestinal microflora on the sensitivity of the colon to the carcinogenic effect of the intrarectal instillation of AOM in germ-free, conventional, and *Clostridium perfringens*-monocontaminated rats. The number of colon tumors per rat was higher in germ-free and monocontaminated rats than in the conventional controls. Monocontaminated rats developed more neoplasms than did germ-free rats. It was speculated that, in germ-free rats, there might be an increased absorption of AOM leading to an altered metabolism in the liver with increased amount of carcinogen and/or cocarcinogen supplied to the target organ. It was also speculated that the increased incidence of tumors in the monocontaminated group might be caused by the strong β-glucuronidase activity of *C. perfringens*.

The plant product, cycasin, after p.o. intake reaches the large intestine where the bacterial enzyme, β-glucosidase, hydrolyzes cycasin to MAM, the active metabolite (27, 28, 30, 34). Thus, the intestinal microflora play a modifying role in colon carcinogenesis not only by liberating active metabolite but also by supplying promoters or accelerators which act on colonic mucosa.

AOM and DMH do not produce tumors at the site of injection, so it is presumed that metabolism is required for activation. AOM is hydroxylated to MAM in the liver, and it has been postulated by Weisburger (43, 44) that this is conjugated with glucuronic acid in the liver and excreted in the bile. The bacterial flora of the intestine may then convert this compound into aglycone MAM by the action of β-glucuronidase. The effect of dietary fiber on the incidence of colorectal tumors due to DMH was studied by Bauer *et al.* (5). In the wheat bran and carrot fiber group, the incidence of colorectal tumors was almost the same as in the fiber-free diet group. The citrus pectin group, however, had a significantly higher incidence of colorectal tumors, and dietary pectin induced a 10-fold increase in fecal β-glucuronidase activity but did not alter this activity in the bowel wall.

Matsumoto *et al.* (32) synthesized the glucuronic acid conjugate of MAM and found that this compound was mutagenic to *Salmonella typhimurium* when preincubated with Escherichia coli β-glucuronidase but not when preincubated with bovine liver β-glucuronidase.

The effect of colostomy on intestinal carcinogenesis by AOM (10) and MAM acetate (31) has been studied. Animals with single-barreled colostomy developed tumors in the colon distal to the colostomy where the mucosa had no contact with the fecal stream. These results indicate that carcinogens can probably reach the intestinal mucosa via the vascular system as well as by biliary transport.

In the case of bladder cancer induction, it is thought that urinary β-glucuronidase hydrolyzes the conjugates liberating 2-amino-1-naphthol or 2-naphtholhydroxylamine, which are the carcinogenic metabolites of 2-naphthylamine. Boyland *et al.* (7) therefore used β-glucuronidase inhibitor to prevent bladder cancer induction as follows: of 8 dogs treated with 2-naphthylamine, 4 were given 1,4-saccharolactone, a potent β-glucuronidase inhibitor. All dogs developed carcinomas but later in the saccharolactone-treated animals than in the controls.

In carcinogenesis induced by chemical agents, it is thought that the carcinogenic action can be prevented by the removal, inhibition, or inactivation of either the carcinogenic metabolites or the enzyme which metabolizes the carcinogen.

We induced colon carcinomas with AOM and studied the effect of β-glucuronidase inhibitor on them. Fewer tumors were found in the rats given C-GAL at the same time as AOM, but those given C-GAL after AOM treatment had slightly more colon tumors than did the controls. The inhibition of tumor formation was strong in the proximal half but insignificant in the distal half of the colon of rats treated with C-GAL. The reason for this difference is not clear. We suspect that it may be due to the concentration of free carcinogen as well as the proportion of free carcinogen to conjugated carcinogen in the intestinal tract. Group B rats had double the number of tumors of Group C animals. In the proximal half of the colon, the number of tumors in both groups was almost the same, but in the distal half, the number of tumors in Group B was more than twice that in Group C. Group D rats showed that C-GAL had no effect on the number of tumors in the proximal half of the colon but did increase those in the distal half when given after AOM treatment. These results indicated that C-GAL or its metabolites may promote or accelerate colon carcinogenesis due to AOM provided chemically or nutritionally, which becomes highly concentrated in the distal half of the colon.

The diameters of the colon tumors varied from 0.1 to 3.1 cm, and the distribution of size was almost the same in each group. This indicates that C-GAL probably has no effect on tumor growth.

This investigation suggests that bacterial β-glucuronidase may play an important role in colonic carcinogenesis due to AOM, which apparently requires metabolic activation.

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