Inhibitory Effect of Fumaric Acid on 3'-Methyl-4-(dimethylamino)azobenzene-Induced Hepatocarcinogenesis in Rats

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Fumaric acid (FA) suppressed the carcinogenesis in the liver of rats fed 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB), and a study was performed to examine the effect of FA on deoxyribonucleic acid (DNA) synthesis and subcellular structures of hepatocytes under the anticarcinogenic regimen. Male Donryu strain rats were given 3'-Me-DAB by being fed a diet containing 0.06% 3'-Me-DAB for 50 d. They were then given a diet containing 1% FA and drinking water containing 0.025% FA for 53 to 69 weeks. Hepatocytes were isolated from the liver by the collagenase perfusion method and placed in culture, and their activity for DNA synthesis was measured in terms of the incorporation of [^{3}H]dThd into DNA. An enhanced DNA synthesis of hepatocytes was noted in the rats given FA, indicating that FA enhanced the proliferation of hepatocytes to counteract the carcinogenic effect of 3'-Me-DAB. An electron microscopic examination indicated that the distribution of subcellular organella was almost normal in the FA-treated hepatocytes.

Keywords fumaric acid; 3'-methyl-4-(dimethylamino)azobenzene; rat hepatocytes; DNA synthesis; anticarcinogenesis

Introduction

Previously we indicated that the extract of *Capsella bursa-pastoris* (Cruciferae) herb has various kinds of pharmacologic activities such as diuretic, oxytocic, anti-inflammatory, anti-ulcerative and anticarcinogenic.[1-4] Later we isolated and identified fumaric acid (FA) as the component of the herb responsible for inhibiting the growth of subcutaneously transplanted Ehrlich tumors in mice[5] or gastric ulceration in rats.[6]

Recently, we found that FA reduced the toxicity of mitomycin C in mice,[7,8] aided in the recovery of cultured cells exposed to mitomycin C and some carcinogens,[9] and suppressed the carcinogenesis in forestomachs and lungs of mice fed a nitrofuran carcinogen[10] as well as the hepatocarcinogenesis in rats fed 3'-methyl-4-(dimethylamino)-azobenzene (3'-Me-DAB).[11] An attempt was made to elucidate the mode of action of FA and it was found that FA enhanced *de novo* deoxyribonucleic acid (DNA) synthesis of rat hepatocytes to counteract the acute hepatotoxicity of mitomycin C.[12] The present study was performed to examine the effect of FA on DNA synthesis and subcellular structures of hepatocytes in 3'-Me-DAB-induced hepatocarcinogenesis in rats.

Materials and Methods

Chemicals Collagenase type I was purchased from Sigma Chemical Co., St. Louis, Mo. Williams' medium E and fetal bovine serum were purchased from Grand Island Biological Co., Grand Island, N. Y. [Methyl-^{3}H]dThd (50 Ci/mmol) was purchased from Amersham International plc, Buckinghamshire, England, and Aquasol-2 was purchased from New England Nuclear, Boston, Mass.

Animals and Feed Male Donryu strain rats (Nippon Rat Co., Saitama), 2 months of age, were used. Commercial diet CE-2 (CLEA Japan Inc., Tokyo) was used as the basal diet, and an experimental diet containing either 0.06% 3'-Me-DAB or 1% FA was also prepared by the same manufacturer. FA was purchased from Nakarai Chemicals, Ltd., Kyoto, and was dissolved in the rats' drinking water at a final concentration of 0.025%. The animals were housed in an air-conditioned room kept at 23 °C and 60% relative humidity with a 12-h light and 12-h dark cycle. The feeding schedule is shown in Fig. 1. Rats weighing 200—250 g were given the diet containing 0.06% 3'-Me-DAB for 50 d, after which they were divided into groups 1 and 2. Rats of group 1 were then given basal diet and ordinary drinking water for 53—69 weeks. Rats of group 2 were given a diet containing 1% FA and drinking water containing 0.025% FA for the same period. Rats of the control group were maintained on basal diet and ordinary drinking water throughout the feeding period.

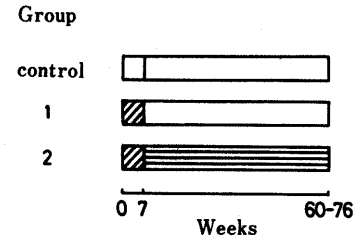

Fig. 1. Dose Schedule of 3'-Me-DAB and FA

▨, given a diet containing 0.06% 3'-Me-DAB; ▬, given a diet containing 1% FA and drinking water containing 0.025% FA; □, given a basal diet.

Culture of Hepatocytes Hepatocytes were isolated from rat liver by the collagenase perfusion method of Berry and Friend.[13] Cell yields were 3—5 × 10^8 with 80—90% viability as judged by trypan blue dye exclusion. The hepatocytes were finally suspended in Williams' medium E supplemented with glutamine (0.6 mg/ml) and fetal bovine serum (10%) at the concentration of 5 × 10^5 cells/ml, plated in a 60-mm (4 ml) polystyrene Petri dish (Corning Glass Works, Corning, N. Y.), and cultured in humidified air containing 5% CO_2 in a CO_2 incubator (type CO-MINI, Tokiwa Kagaku Kikai Co., Ltd.). Penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml) were added to Ca^{2+}-free Hanks' and culture medium.

Measurement of Incorporation of [^{3}H]dThd into DNA The cells were plated in 60-mm dishes, pre-incubated for 1.5 h, and then exposed to [^{3}H]dThd (5 μCi/ml) for 2 h. At the termination of exposure, the cells were transferred to 15-ml centrifuge tubes, and precipitated by centrifugation at 2500 rpm for 5 min. The precipitates were washed once with 0.9% NaCl solution, once with 0.5 M $HClO_4$, and twice with 0.2 M $HClO_4$. The precipitates were dissolved in 4 ml of 0.3 M KOH to hydrolyze ribonucleic acid (RNA) at 37 °C for 1 h, cooled on ice, acidified with 2.5 ml of 1 M $HClO_4$, and centrifuged.[7,14] The supernatants were discarded, and the precipitates were washed twice with 0.5 M $HClO_4$, suspended in 2 ml of 1 M $HClO_4$, heated at 70 °C for 20 min, cooled on ice, and centrifuged. The final supernatants were used to determine DNA content by the use of the diphenylamine reagent[15] and radioactivity as described previously.[7] The radioactivity was counted in a Packard Tri-Carb model 3255 liquid scintillation counter.

Electron Microscopy Isolated hepatocytes were fixed in a formaldehyde–glutaraldehyde fixative for 12 h at 5 °C and then post-fixed in 1% OsO_4 for 2 h at room temperature. After dehydration with a graded series of ethanol, the pieces were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate solution. The stained speciments were examined in Hitachi H700H electron microscope.

Results and Discussion

A rat was taken from each of the 3 groups at a specified day between 60 and 76 weeks after the start of the experimental feeding (Fig. 1). Then hepatocytes were iso-

TABLE I. Effect of 3′-Me-DAB and FA on the Incorporation of [^{3}H]dThd into DNA of Hepatocytes

Group	No. of rats	Time in dose schedule (week)	[^{3}H]dThd incorporation into DNA (dpm/μg DNA)
Control	4	60—62	602±132
1	4	60—62	680± 80
2	4	60—62	1252±240[a]
Control	4	74—76	533±158
1	4	74—76	510± 95
2	4	74—76	1130±311[b]

a, b) Values are means±S.D. Student's *t* test: *a*) $p<0.01$ *vs.* control group; *b*) $p<0.05$ *vs.* control group.

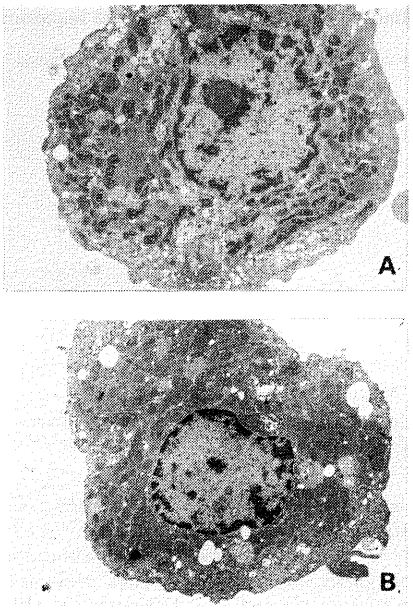

Fig. 2. Isolated Hepatocyte from a Rat of Group 1 (A) or Group 2 (B) (×6000)

lated from each liver by the collagenase perfusion method and cultured to incorporate [^{3}H]dThd into DNA. A part of the hepatocytes was fixed for electron microscopy. As shown in Table I, an enhanced DNA synthesis of hepatocytes was noted in rats given FA (group 2), indicating that FA enhanced the proliferation of hepatocytes to counteract the carcinogenic effect of 3′-Me-DAB. The electron microscopy of hepatocytes revealed some deleterious alterations brought about by the ingestion of 3′-Me-DAB (Fig. 2A, group 1); mitochondria were atrophic and small in number, rough endoplasmic reticulum showed irregular arrangement and poor development, and the nuclear outline was irregular. The nucleolus was increased in size. In the hepatocytes from rats given FA, the distribution of subcellular organella showed no definite changes (Fig. 2B, group 2); mitochondria showed clear crista and a dense matrix, the rough endoplasmic reticulum was well developed, and the nuclear margin was round and smooth. The heterochromatin was well developed and the size of the nucleolus was almost normal.

The ability of FA to enhance DNA synthesis of hepatocytes is interesting in connection with the anticarcinogenic activity of this acid. Previously we showed that FA enhanced *de novo* DNA synthesis of hepatocytes in the liver to counteract the toxicity of a hepatotoxin, mitomycin C or aflatoxin B_1, whereas such activity of FA was not seen in hepatic cancer cells, a 3′-Me-DAB-induced transplantable cell line growing in the abdominal ascites.[12] One possible mode of action of FA is that FA stimulates the proliferation of normal or intact hepatocytes in the liver and this leads to elimination of the altered or precancerous cells.

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