

Modulation of Tumor Promotion in Liver Carcinogenesis

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Earlier, we demonstrated that feeding to rats a diet devoid of choline, a lipotropic factor, markedly enhances hepatoma induction by several chemical carcinogens, and that the diet acts as a strong promoter of the evolution of initiated cells to foci of altered hepatocytes. The ability of several factors to modulate the action of the choline-devoid (CD) diet as a promoter was investigated by quantitating the foci of γ -glutamyl transpeptidase-positive hepatocytes developed in rats exposed to a single injection of diethylnitrosamine. Addition to the diet of phenobarbital (PHB) resulted in a promoting action stronger than those of the CD diet or of PHB alone. Two other barbiturates, amobarbital (AMB) and pentobarbital (PTB) exerted an effect similar to that of PHB, while barbituric acid (BA) had no effect. In other studies, lowering the fat content of the CD diet reduced its efficacy as a promoter, while the addition of a hypolipidemic agent, BR931, 4-chloro-6-(2,3 xylidino)-2-pyrimidinylthio (*N*- β -hydroxy-ethyl)acetamide, completely abolished the promoting action of the CD diet. In rats not exposed to carcinogen, feeding the CD diet caused a marked enhancement of liver DNA synthesis and of cell proliferation. Inclusion of PHB, PTB or AMB in the CD diet inhibited these effects, while BA exerted no inhibition. The increased rate of DNA synthesis and cell proliferation in the liver were not affected by the level of fat in the CD diet. These results suggest that beside a stimulation of liver cell proliferation, other factor(s) determine the efficacy with which a CD diet exerts its promoting action.

Introduction

Earlier, we demonstrated that feeding a diet devoid of choline, a lipotropic factor, markedly enhanced liver tumor induction in rats by several chemical carcinogens either included in the choline-devoid (CD) diet or administered while rats were on the CD diet (1-3). Further studies showed that feeding the CD diet strongly promotes the evolution of liver cells, initiated by chemical carcinogens, to foci of γ glutamyl transpeptidase (GGT +) positive hepatocytes (4) and the evolution of the foci to hepatomas (5).

Thus, the action of a CD diet is very similar to that of phenobarbital (PHB) which has been shown to be also an effective promoter of liver carcinogenesis (6-9).

More recently, we found that inclusions of PHB in a CD diet resulted in a synergistic effect of the two agents (10). Indeed, in rats exposed to a single injection of diethylnitrosamine (DEN), foci of GGT +

hepatocytes developed in a number greater than the sum of those observed in rats treated with either agent alone. Studies on the mechanisms by which certain agents modulate the efficacy of individual promoting agents may become useful in analyzing the critical features of tumor promotion in the liver. In this paper, we describe three other conditions modulating the promoting action of a CD diet, and present preliminary data on possible mechanisms underlying their action.

Materials and Methods

Male Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, PA) weighing 150-165 g were used in all experiments. The animals were housed in pairs in metal cages in a room with temperature, humidity and light controls, and were given laboratory chow (Purina, Ralston Purina Co., St. Louis, MO) and water *ad libitum*. Purified CD and choline supplemented (CS) diets were prepared as previously described (11). These diets contain 14% fat. A low-fat CD diet was prepared by reducing the levels of corn oil and of Premix to 1 and 3%, respectively,

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while keeping the same relative proportions of the other ingredients. PHB, pentobarbital (PTB) and amobarbital (AMB) at a level of 0.06% and barbituric acid (BA) at a level of 0.04% were incorporated into the CD and CS diets at the expense of sucrose. All agents were from Sigma Chemical, St. Louis, MO. A hypolipidemic agent, 4-chloro-6-(2,3-xylidino)-2-pyridimidinylthio(*N*- β -hydroxyethyl)acetamide (BR931) (LPB Instituto Farmaceutico, Milan) was kindly supplied by Dr. J. Reddy, Northwestern University, Chicago, and was incorporated into the CS and CD diets at a level of 0.2%.

For the assays of the induction of foci of GGT + hepatocytes, rats were given a single intraperitoneal injection of DEN (Aldrich Chemical Co., Madison, WI) at doses of 40 or 50 mg/kg, 18 hr after a partial hepatectomy, or of 160 mg/kg without a partial hepatectomy. One week later, the animals were divided into groups, each of which was fed one of the experimental diets. The animals were sacrificed 4, 6 or 7 weeks thereafter, and liver sections were prepared for quantitative of foci of GGT + hepatocytes as previously described (4).

For determination of DNA synthesis, each animal received a single IP dose of 50 μ Ci/100 g body weight of [³H]-thymidine (20 Ci/mmol, New England Nuclear Corp. Boston, MA), 2 hr before killing by decapitation. The liver was quickly excised, rinsed in cold saline, blotted dry, weighed, and a 10% homogenate was immediately prepared in cold 0.6 N perchloric acid (PCA). DNA was selectively hydrolyzed, and radioactivity was determined as previously described (12).

For counting the number of mitosis, blocks of liver were fixed in a Stieve's solution, and 6 μ m

thick sections were stained with hematoxylin and eosin. Entire sections of the right and left lobes were examined at a 400 \times magnification, and hepatocytes in mitosis were counted. The surface area of the sections was calculated with the aid of a planimeter, and the number of mitosis per square centimeter of section were calculated. The results of all experiments were evaluated statistically by Student's *t* test, and differences between means were regarded as significant if $p < 0.05$.

Results

Table 1 summarizes the results obtained in two separate experiments in which barbiturates were tested for their ability to promote the evolution of liver cells, initiated with a single injection of DEN, to foci of GGT + hepatocytes. PHB, PTB, and AMB, added to the CS diet, exhibited a comparable degree of promotion, but barbituric acid showed no promoting action. In agreement with our earlier observation (10), addition of PHB to the CD diet resulted in a synergistic effect in as much as the number of foci was greater than the sum of those observed in rats treated with the CD diet alone or the CS + PHB diet. Addition of PTB to the CD diet also resulted in a synergistic effect, while the combination of AMB and CD diet showed only an additive effect. On the other hand, inclusion of BA in the CD diet resulted in no increase in the promoting action of the CD diet.

The effect of varying the fat content of the CD diet was next investigated. As shown in Table 2, the number of foci observed after 4 and 6 weeks of feeding the high-fat CD diet was significantly higher

Table 1. Effect of barbiturates on the induction of GGT-positive foci in the liver.

Experiment and diet ^a		No. foci/cm ^{2b}	Diameter, μ m
I	CS	1.56 \pm 1.1	156.3 \pm 6.3
	CS + amobarbital	11.4 \pm 3.3*	159.1 \pm 6.1
	CS + pentobarbital	13.4 \pm 1.6*	160.4 \pm 5.2
	CS + phenobarbital	14.1 \pm 2.3*	163.7 \pm 6.3
	CD	10.4 \pm 1.0	239.9 \pm 14.6
	CD + amobarbital	23.5 \pm 2.5***†	307.4 \pm 17.4‡
	CD + pentobarbital	42.7 \pm 7.6***†	362.8 \pm 27.5‡
	CD + phenobarbital	35.4 \pm 3.9***†	293.3 \pm 16.0‡
II	CS	0.6 \pm 0.4	188.6 \pm 17.8
	CD	17.2 \pm 3.0	323.9 \pm 19.1
	CS + barbituric acid	0.9 \pm 0.4	158.6 \pm 5.1
	CD + barbituric acid	22.7 \pm 2.8	293.4 \pm 10.1

^a In both experiments, rats were initiated with a single injection of DEN (40 mg/kg), 18 hr after a partial hepatectomy. In experiment I the rats were killed after 6 weeks of the dietary promotion, and in experiment II after 7 weeks.

^b Each figure represents the mean \pm SE of four to six rats.

* $p < 0.01$ against the CS group.

** $p < 0.01$ against the CD group.

† < 0.05 against the CD group.

‡ < 0.05 against the CD group and $p < 0.01$ against the corresponding CS groups.

Table 2. Induction of GGT + foci in the liver of rats fed high or low fat CD diet.^a

Promotion	Time, weeks	Body weight, g ^b	Liver weight, g ^b	No. of foci/cm ^{2b}	Size, mm ^b
CD-high fat	4	302.8 ± 14.8	16.4 ± 1.0	17.7 ± 6.4	250 ± 18
CD-low fat	4	297.6 ± 6.3	17.2 ± 1.9	7.7 ± 3.1	223 ± 16
CD-high fat	6	392 ± 19.5	22.0 ± 1.6	27.4 ± 4.2	358 ± 54
CD-low fat	6	413 ± 20.0	23.8 ± 1.8	15.8 ± 2.5	236 ± 15

^aRats were initiated with a single injection of DEN (50 mg/kg) 18 hr after a partial hepatectomy. Promotion was started 1 week after initiation, and rats were killed 4 and 6 weeks thereafter.

^bEach figure represents the mean ± SE of five rats.

Table 3. Effect of BR931 [4-chloro-6-(2,3-xylydino)-2-pyrimidinylthio-(N-β-hydroxyethyl)acetamide] in the induction of GGT+ foci in the liver.

Initiation ^a	Promotion ^b	No. of foci/cm ^a	
		4 weeks promotion	6 weeks promotion
Saline	CS + BR931	0	0
Saline	CD + BR931	0	0
DEN	CS	1.3 ± 0.6	0.3 ± 0.2
DEN	CD	6.9 ± 1.5	16.1 ± 4.0
DEN	CS + BR931	0	0
DEN	CD + BR931	0	0

^aRats were initiated with a single injection of DEN (160 mg/kg). Each group consists of seven or eight rats.

^bPromotion was started 1 week after the initiation.

than that after feeding the low fat CD diet. At each point, body and liver weights of the animals showed no difference between the two groups.

The effect of BR 931 on the induction of foci of GGT-positive hepatocytes was examined in rats initiated with a single injection of DEN (160 mg/kg) without a prior partial hepatectomy (Table 3). After 4 and 6 weeks of dietary promotion, BR 931, added to the CD diet completely abolished the promoting action of the CD diet.

Earlier we demonstrated that feeding a CD diet to rats causes an increase of liver DNA synthesis and of cell proliferation (hepatocyte mitosis) over those present in rats fed the CS diet, and that inclusion of PHB in the CD diet inhibits such a stimulation (12, 13).

The effects of other barbiturates included in the diet on the stimulation of liver DNA synthesis and liver cell proliferation induced by the CD diet were investigated, and the results are shown in Figures 1 and 2. PTB inhibited DNA synthesis and cell proliferation, and the degree of inhibition was comparable to that exerted by PHB. AMB, as judged by mitotic counts, showed a lesser degree of inhibition (Fig. 2), and BA had no significant effect on the CD diet-induced stimulation of DNA synthesis and cell proliferation.

Comparative studies of the rate of liver DNA synthesis and cell proliferation in rats fed a high or low fat CD diet were also performed, and the re-

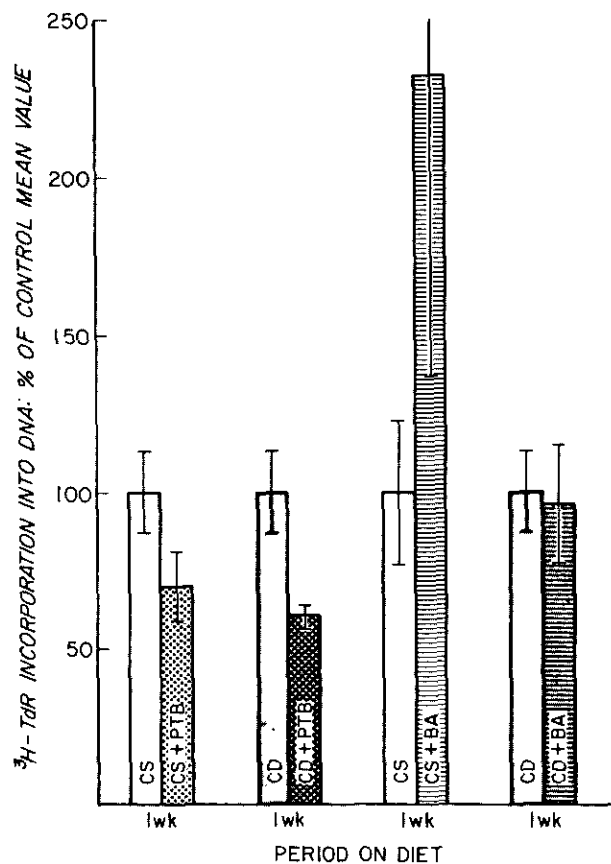


FIGURE 1. Effect of feeding for 1 week a choline-supplemented (CS) or choline-devoid (CD) diet and the same diets containing 0.06% pentobarbital (PTB) or 0.04% barbituric acid (BA) on incorporation of ³H-thymidine into liver DNA. Four rats in each group received a single IP dose of ³H-thymidine, 50 μCi/100 g body weight, 2 hr before killing by decapitation. The radioactivity in DNA was determined as described in the text and the results are expressed as mean % ± SE of control (CS or CD) value; *p* < 0.05 between the CD and CD + PTB.

sults are shown in Table 4. Comparable levels of DNA synthesis and cell proliferation were observed in rats fed the high and low fat CD diets for 1 and 2 weeks, but the levels were significantly higher than those present in the liver of rats fed a CS diet.

Discussion

Experimental studies of liver carcinogenesis have recently confirmed the validity of the concept of a two stage development of hepatomas, and a growing number of promoters of liver carcinogenesis have been identified in the past few years (14). However, the nature of and the mechanism underlying tumor promotion in the liver are poorly understood. In our laboratory, we have developed a dietary means of promotion of hepatoma induction in rats, namely feeding a diet devoid of choline (4, 5).

The present study demonstrates three conditions which modulate the promoting efficacy of the CD diet, one being stimulatory and the others being either partially or completely inhibitory. Analysis of the mechanism of action of these modulated systems may assist in elucidating the critical features of tumor promotion in the liver.

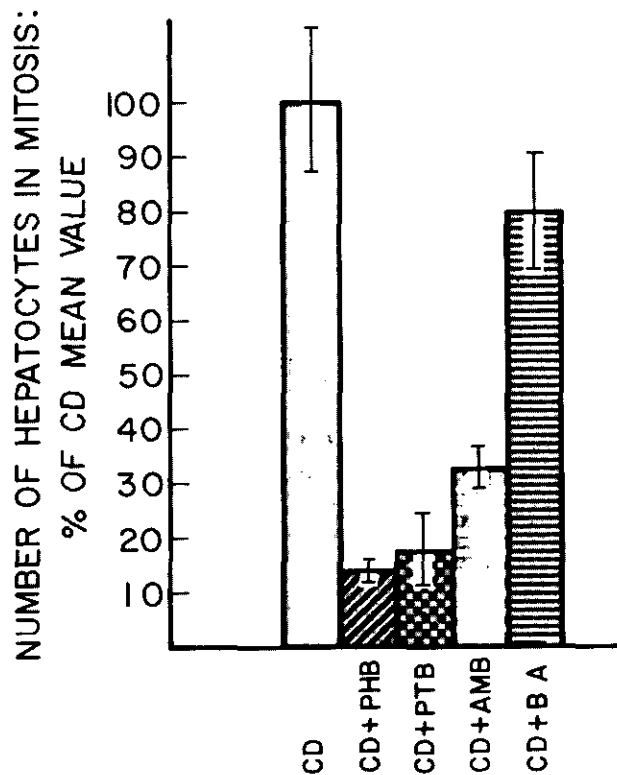


FIGURE 2. Effect of feeding for 1 week a choline-devoid (CD) diet or the CD diet containing 0.06% phenobarbital (PHB), pentobarbital (PTB), amobarbital (AMB) or 0.04% barbituric acid (BA) on the number of hepatocytes in mitosis. Mitoses were counted on hematoxylin eosin-stained liver sections of each animal at a $400\times$ magnification, and the number of mitoses/cm² of section was calculated. The results are expressed as % of CD mean value \pm SE of four rats. $p < 0.01 =$ CD vs CD + PHB, CD + PTB, CD + DTB and CD + AMB; $p < 0.01 =$ CD + AMB vs. CD + PHB and CD + PTB.

As a step toward achieving this goal, we studied and compared the three conditions as promoters of the emergence of foci of altered hepatocytes, and their effects on liver cell DNA synthesis and cell proliferation. Using three structurally related barbiturates, we confirmed and extended our earlier observations that inclusion of PHB into a CD diet markedly enhances the promotion action of the diet (10).

PTB and AMB, when added to the CS diet, were shown to be effective in promoting the evolution of initiated cells to foci of GGT-positive hepatocytes. However, BA, at a level equimolar to the other barbiturates, had no promoting effect. These findings indicate that the promoting activity of barbiturates is dependent on biological effects due to the presence of different alkyl groups at the C-5 position of BA. As in the case of PHB, inclusion of PTB or AMB into the CD diet resulted in an enhanced promoting activity, though the former exerted a stronger enhancement than the latter. It is of interest to note that the degree of stimulation of promotion correlates in part with the degree with which these barbiturates inhibit the CD diet-induced DNA synthesis and cell proliferation in the liver. Indeed, PTB inhibited DNA synthesis and cell proliferation as effectively as PHB, while AMB, as judged by mitotic counts, showed a lesser degree of inhibition. BA, on the other hand, had no significant effects on either DNA synthesis and cell proliferation or promotion. These observations support our earlier suggestion that the suppression of cell growth of the noninitiated population of cells in an initiated liver may result in a selective growth stimulation of the initiated cells, though direct evidence is lacking (13).

The results obtained with CD diets containing a high or low level of fat, are consistent with a num-

Table 4. Liver DNA synthesis and hepatocyte mitosis in rats fed high fat-CS, high fat-CD or low fat-CD diet.

Diet	Time on diet, weeks	³ H-Thd incorporation, dpm/ μ g DNA ^b	Hepatocyte mitosis, no. of hepatocytes/cm ² liver ^{a,b}
CS-high fat	1	71.1 \pm 12.6 ^b	6.1 \pm 3.4
CD-high fat	1	361.0 \pm 114.8*	70.7 \pm 10.6*
CD-low fat	1	335.0 \pm 27.7*	60.8 \pm 12.9
CS-high fat	2	76.3 \pm 42.2	1.7 \pm 1.7
CD-high fat	2	142.1 \pm 67.6**	36.4 \pm 6.8*
CD-low fat	2	160.3 \pm 33.8**	52.8 \pm 12.5*

^aNumber of hepatocytes in mitosis per square centimeter of liver sections.

^bEach figure represents the mean \pm SE of three or four rats.

* $p < 0.01$ against the CS group.

** $p < 0.05$ against the CS group.

ber of observations in literature that a high fat content in the diet has a general enhancing effect on the development of spontaneous tumors, as well as on the induction of tumors by chemicals in experimental animals (15, 16).

Using a combined lipotrope-deficient diet, Rogers and her associates have demonstrated that the high fat content in the diet markedly enhances hepatocarcinogenesis by a variety of carcinogens (17, 18). Even though the role played in tumor promotion by both quantity and quality of dietary fat appears to be complex, it was of interest to find in the present study that there is no correlation between efficacy of the promotion and degree of stimulation of liver cells proliferation with CD diets containing different amounts of fat. Enhanced liver cell proliferation induced by a partial hepatectomy or a necrogenic dose of carbon tetrachloride has been shown to accelerate the development of liver tumors in rats or mice initiated with a variety of carcinogens (19-22). However, our findings suggest that the general properties of enhanced cell proliferation per se are not the sole factors in determining the efficacy of tumor promotion in the liver and that other factors must be involved.

The apparent lack of the promoting activity by BR 931, as assayed in our test system, is of considerable interest. BR 931, one of many hypolipidemic agents which stimulate microbody proliferation in the liver and induce hepatomegaly, has been shown to induce hepatomas in rats and mice after long term administration (23). In our system, RB 931 showed no promoting activity when added to the CS diet, and completely abolished the promoting activity of the CD diet. As many other hypolipidemic agents, BR 931 has been shown to stimulate liver cell proliferation without inducing cells necrosis (24).

Although we have not yet analyzed cell proliferation kinetics in the liver of rats fed BR 931 with our dietary regimens, this agent appears to provide a unique situation in which stimulation of liver cell proliferation may not promote the evolution of initiated cells to foci of GGT positive hepatocytes. Obviously, further studies are needed to answer this intriguing question.

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