Chemical carcinogenesis in methyl-deficient rats

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Introduction

The basis of extensive research into chemically induced and "spontaneous" tumorigenesis in rodents deficient in dietary methyl supply is the report in 1946 of the development of hyperplastic nodules and hepatocellular carcinoma in lipotrope-(methyl)-deficient rats.[1] The authors of that report summarized their results as follows:

"It appears that marked accumulation of fat in the liver cells resulting from an insufficiency of choline, or more specifically of labile methyl groups, initiates a sequence of responses which result in profound changes in the structure and behavior of cells."[1]

They then described the changes with emphasis on hyperplasia, increased mitoses, and dysplasia of hepatocytes culminating in carcinoma.

Subsequent studies in several different lipotrope-(or methyl-)deficient models demonstrated that the deficiency increased the sensitivity of the liver to virtually every chemical hepatocarcinogen tested (*Table 1*).[2-7] These results and the identification of carcinogenic mycotoxins led to the assumption that Copeland and Salmon had detected the increased sensitivity of the deficient liver to chemical carcinogens, and that their diets were contaminated by carcinogenic mycotoxins, including aflatoxins. In 1983, however, Mikol et al.[8] demonstrated unequivocally that the deficiency itself induced hepatocellular carcinoma in the absence of exposure to any known carcinogen. This result has been confirmed in other laboratories.[9,10] Mikol et al.[8] also reported a significant correlation between the incidence of diethylnitrosamine-induced hepatocellular carcinoma and the amount of reduction of dietary labile methyl supply.

Labile methyls are supplied primarily by choline and methionine in the diet and can be regenerated endogenously through folate and vitamin B_{12} pathways. Severity of the dietary deficiency can, therefore, be increased by reducing folate and vitamin B_{12}, as well as by reducing choline and methionine. In addition, deficiency of choline and methionine alters folate metabolism and storage.[11]

The observations of the effect of lipotrope deficiency on chemically induced and "spontaneous" hepatocarcinogenesis have led to experiments that have greatly advanced understanding of carcinogenesis. They have, in addition, advanced understanding of the roles of hyperplasia and preneoplastic, enzyme-altered, or hyperplastic foci in hepatocarcinogenesis.[12-18] They have led to increased knowledge of labile methyl nutrition, metabolism, and effects on carcinogenesis.[11-19]

Labile methyl deficiency and hepatocarcinogenesis

Deficiency of supply of labile methyl groups is the only dietary deficiency known that by itself appears to be carcinogenic. Its effect may be found to be promotion of the carcinogenic action of one or more endogenous or exogenous agents, but, if so, they remain to be identified. The deficiency can induce severe liver damage and cirrhosis, but its effect on chemical carcinogenesis in the liver does not require deficiency severe enough to induce cirrhosis.[3,5,12] In fact, the enhancement of carcinogenesis may even be reduced by severe deficiency.[17]

Because the deficiency apparently can induce hepatocellular carcinoma, an initiating effect may be assumed unless one argues that unknown initiators are responsible. There are studies in the diethylnitrosamine (DEN) model that clearly demonstrate that the deficiency also is a promoter.[5,15,18] The deficient diet has many biochemical and molecular effects in the liver.[11-19] It alters carcinogen metabolism, but the results are not always readily interpretable with respect to ultimate carcinogenesis. For example, aflatoxin B_1 (AFB_1)-induced hepatocarcinogenesis and hepatic AFB_1-DNA adducts are increased in deficient rats given repeated doses of AFB_1.[20] Assay of mutagenic activity of urine from rats given AFB_1 indicated that deficient rats excreted a

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Table 1 Lipotrope deficiency[a] and hepatocarcinogenesis

Carcinogen[b]	Rats	Tumor incidence (%)		Reference
		Control	Deficient	
AFB1	F344 Male	11	87	Rogers, 1975
AAF	SD Male	19	41	Rogers, 1975
	F344 Female	36	72	Rogers and Newberne, 1980
DDCP	SD Male	29	63	Rogers, 1975
DEN	SD Male	24	60	Rogers et al., 1974
DBN	SD Male	24	64	Rogers et al., 1974
Ethionine	SD Male	0	50–80	Shinozuka et al., 1978a
L-azaserine	Wistar Male	0	67	Shinozuka et al., 1978b
L-azaserine	Lewis Male	11	65	Rogers et al., 1987
None	F344 Male	0	43	Miko et al., 1983
None	F344 Male	0	26	Chandar and Lombardi 1988
None	F344 Male	0	15	da Costa et al., 1993

[a]In rats deficient during and after carcinogen exposure or only during exposure.
[b]Abbreviations: AFB, aflatoxin B; AAF, N-2-fluorenylacetamide; DDCP, 3,3-diphenyl-3-dimethylcarbamoyl-1-propyne; DEN, diethylnitrosamine; DBN, dibutylnitrosamine.

greater fraction of the AFB_1 as activated, glucuronide-bound material than rats fed the control diet; the controls excreted primarily unchanged AFB_1.[21] These results are consistent with the observed increase in carcinogenicity of AFB_1 and indicate a primary effect at initiation. However, in vitro metabolism of AFB_1 to microbial mutagens was reduced in liver preparations from deficient rats compared with control rats.[21]

Blood clearance of DEN but not of dibutylnitrosamine (DBN) was reduced in deficient rats, although both compounds are more effective hepatocarcinogens in deficient rats.[22] This observation is explained by studies in the hepatocarcinoma models induced by single doses of DEN or DBN in which there is clear evidence that the deficiency has a promoting effect.[8,15,18] No evidence of an effect of deficiency on promotion of I-azaserine-induced tumors was detected by feeding the deficient diet after carcinogen exposure,[7] but the exposure extended over 10 weeks. Therefore, the deficient diet was not fed until the rats were nearly 4 months old. In the single dose carcinogen models, deficiency is induced at about 6 weeks of age. Because the severity of methyl deficiency declines with increasing age of the rat, the absence of a demonstrable effect on carcinogenesis when the deficiency was instituted so late may have been due to the mildness of the deficiency induced. Adaptation to methyl deficiency with increasing age has been recognized for many years; it is evident in the decrease in hepatic lipid with prolonged feeding and, in a recent study, in the recovery of hepatic folate stores in prolonged deficiency.[23]

While the protective effect against chemical hepatocarcinogenesis of a methyl-sufficient diet is clear, a benefit of supplementation beyond the nutritional requirement has not been demonstrated in rats except in the case of ethionine, which is directly antagonized by methionine.[24,25] Addition of 1.5% DL-methionine or 1% choline chloride to a nutritionally complete natural-product diet fed to rats after a single dose of DEN did not reduce hepatocellular carcinoma (HCC) incidence compared with the incidence in rats fed the unsupplemented diet.[26] In the procarbazine HCL (PCZ)-induced mammary tumor model in female rats discussed below, no effect of hypersupplementation with choline and methionine was detectable, although tumorigenesis did show a dose response to dietary methyl supply at levels below and up to the required level.

Refeeding experiments, that is, experiments in which a lipotrope-sufficient diet is fed after a period of deficiency, have provided data indicating that tumor development may be increased by such treatment compared with development in rats maintained on the deficient diet throughout the experiment. This is of course, different from hypersupplementation of an animal already sufficient in lipotropes. Hepatocarcinogenesis, induced by either the deficiency alone[14] or by carcinogen (l-azaserine) exposure of deficient animals[7] may be increased by refeeding. In the first case, when lipotrope-sufficient diet was fed to deficient rats for 4 months after 12 months of deficiency, they had an HCC incidence of 73% compared with 26% in rats fed the deficient diet for 16 months.[14] In the azaserine experiment, refeeding began 48 hours after a 10-week period of deficiency and I-azaserine exposure. Tumors were found earlier and in higher incidence in refed than in continuously deficient rats; both deficient groups had higher tumor incidences than rats fed control diet throughout the experiment.[7]

Methyl-deficient mice have been studied also, but less extensively than rats. Mice are resistant to the cirrhogenic effects of deficiency, but they appear to respond to deficiency similarly to rats with respect to carcinogenesis. Deficient $B6C3F_1$ mice are more susceptible to AFB_1 carcinogenesis than mice fed a sufficient diet.[27] As had been found in rats, in C3H mice there was no effect of hypersupplementation with methionine or choline on tumors induced by DEN alone or on spontaneous tumors. However, the promoting effect of phenobarbital on spontaneous malignant liver tumors and on DEN-induced malignant tumors was reduced by hypersupplementa-

tion.[28,29] Hypersupplementation of a nutritionally complete diet with methionine and choline significantly reduced AFB_1 carcinogenesis in $B_6C_3F_1$ mice.[30,31]

Labile methyl deficiency and carcinogenesis in organs other than liver

The deficiency enhances 1,2-dimethylhydrazine (DMH) carcinogenesis in colon[3,32] and procarbazine-HCl (PCZ) carcinogenesis in mammary gland,[33*] but its influence in these organs is much less marked than in the liver (*Table 2*). Enhancement by the deficiency of nitrosamine carcinogenesis in esophagus and of azaserine-induced prenoplastic lesions in pancreas has been reported, but is not consistently demonstrable. The result may be dependent on carcinogen dose and route of exposure.[4,6,34,35] DBN, which is a more effective hepatocarcinogen in deficient than in normal rats, is not a more effective bladder, esophagus, or lung carcinogen.[1] Metabolic studies have clarified the relationships between route of exposure, metabolism, and target organ for DBN. Intestinal epithelial cell activation following gastrointestinal absorption produces primarily the major urine metabolite and bladder carcinogen, N-nitrosobutyl-(3-carboxpropyl) amine, while hepatic metabolism produces the alpha-hydroxylated hepatocarcinogenic metabolite.[36,37] Therefore, the bladder carcinogen is produced independently of the liver, and a dietary effect mediated by metabolism would have to be exerted on the intestine.

Of particular interest is the effect of deficiency on mammary gland carcinogenesis. The rat mammary gland is capable of carrying out active triacylglycerol and phospholipid synthesis and secretion. It takes up choline from the blood, metabolizes it, and secretes it and its metabolites into milk. During lactation these pathways are very active.[38] For these reasons, the gland may be expected to be susceptible to lipotrope deficiency because it reduces choline supply and impairs normal fat metabolism in liver and, presumably, in other tissues.

Studies have been performed in lipotrope-deficient[1,3] male and female rats given PCZ, a cancer chemotherapeutic agent that is carcinogenic for mammary glands and hematopoietic tissues of rats. Parallel studies were performed in rats fed a control diet, using low doses of methotrexate (MTX), an antifolate, chemotherapeutic agent, to induce alteration of methyl supply.[33,39*†] Because lipotrope deficiency and MTX alter folate metabolism and create a functional deficiency of folate, it was expected that the sensitivity of the hematopoietic and lymphopoietic tissues to PCZ would be increased. James and Yin[39] reported increased DNA strand breaks in splenic lymphocytes in lipotrope-deficient rats. Branda et al.[40] reported increased mutant frequency in peripheral blood lymphocytes of a women treated for breast cancer with chemotherapeutic regimens that included MTX. The presence of elevated mutations was associated with decreased serum folate levels.

In male rats, the deficiency led to increased PCZ mammary carcinogenesis. MTX gave similar but not

Table 2 Lipotrope deficiency and chemical carcinogenesis in Sprague-Dawley rats in organs other than liver

Organ	Carcinogen[a]	Rats	Control	Deficient	Reference
Mammary gland	PCZ	Male	30	48	Rogers et al., 1990
		Female	69	84	Akhtar, footnote *
Hematopoietic Tissues	PCZ	Male	28	15	Rogers et al., 1990
		Female	0	3	
Zymbal's gland	PCZ	Male	40	25	Rogers et al., 1990
		Female	0	3	
Zymbal's gland	AAF	Male	5	41	Rogers, 1975
Colon	DMH	Male	56 86	83 100	Rogers et al., 1980
Zymbal's gland	DMH	Male	33 75	15 44	Rogers et al., 1980
Forestomach	DDCP	Male	71	53	Rogers, 1975
Forestomach	MNNG	Male	97	100	Rogers, 1975
Urinary bladder	FANFT	Male	53	65	Rogers, 1975
Urinary bladder & Kidney	DBN	Male	84	80	Rogers et al., 1975
Esophagus	DEN	Male	25 35	7 44	Rogers et al., 1975
Esophagus	DBN	Male	12	0	Rogers et al., 1975
Lung	DBN	Male	100	88	Rogers et al., 1975

[a]Abbreviations: AFB, aflatoxin B; AAF, N-2-fluorenylacetamide; DDCP, 3,3-diphenyl-3-dimethylcarbamoyl-1-propyne; DEN, diethylnitrosamine; DBN, dibutylnitrosamine; DMH, 1,2-dimethylhydrazine; FANFT, N-[4-(5-nitro-2-furyl) 2-thiazolyl] formamide; MNNG, N-methyl-N-nitroso-N-nitroguanidine; PCZ, procarbazine HCL

*Akhtar, R. Ph.D., dissertation entitled: Procarbazine mammary carcinogenicity in lipotrope-deficient or methotrexate-treated rats.
†Procarbaxine-induced inhibition of Hepatic Choline Oxidation in Lipotrope-Deficient Rats. R. Akhtar, J.K. Blusztajn, A.E. Rogers.

statistically significant results. There was no evidence of increased leukemia or lymphoma.[33] In female rats, the deficiency increased PCZ carcinogenesis; MTX did not have a detectable effect (*Table 3*).† Deficient, control or MTX-treated rats given normal saline and not given PCZ did not develop tumors.

Abnormalities of hepatic choline metabolism were found in the PCZ-treated rats. The abnormalities were much greater in lipotrope-deficient or MTX-treated rats than in control rats given PCZ (*Table 4*).[33]† In vivo or in vitro exposure of liver to PCZ resulted in accumulation of choline, apparently by blocking the oxidation of choline to betaine. That irreversible reaction is normally the major pathway for utilization of methyl groups from choline for methylation of homocysteine to form methionine. Because the activity of the responsible enzyme, choline dehydrogenase (EC 1.1.99.1), decreases in liver of lipotrope-deficient rats,[11] their greater sensitivity compared with controls rats to PCZ blockage of the enzyme is explained.

Because the mammary gland has active choline metabolism and can convert choline to betaine,[38] PCZ may have similar activity in the gland and disrupt labile methyl metabolism there. There is considerable evidence that hepatocarcinogens disrupt hepatic methyl metabolism, and that this effect may contribute to their carcinogenicity.[19,42] The PCZ studies suggest that the same may be true in the mammary gland.

PCZ produces O^6-methylguanine (O^6-meG) in DNA, presumably the critical lesion for initiation of carcinogenesis, in the mammary gland and other target or nontarget tissues of rats. Activity of the repair enzyme, O^6-alkylguanine-DNA alkyl transferase (AGT), normally is considerably lower in rat mammary gland than in most other tissues, and repair of the O^6-meG lesion in the gland appears low after single or multiple carcinogenic doses of PCZ.[43,44] Effects of the dietary choline-methyl deficiency on AGT are not known.

Acute nonlymphocytic leukemia (ANLL) is by far the most common second tumor in cancer patients treated with PCZ and other chemotherapeutic agents. In PCZ-treated patients, adduct accumulation in leukocytes is inversely proportional to leukocyte AGT levels, a relationship thought possibly important in PCZ-induction of ANLL.[45,46] X-irradiation therapy also may be followed by acute nonlymphocytic leukemia, other lymphohematopoietic tumors, or solid tumors, the site depending on the site of exposure to radiation. Hodgkin's Disease patients, successfully treated in their youth with X-irradiation and chemotherapy, have been a source of concern for some time because of the known leukemogenic potential of the therapeutic agents used and be-

Table 3 Procarbazine carcinogenesis in Sprague-Dawley rats

Diet	Methotrexate	Mammary Gland Tumors		Other Tumors	
		% Incidence	No./Rat	Leukemia, Lymphoma (%)	Zymbal's gland (%)
			Males		
Control	–	30	0.5	28	40
	+	49	0.8	25	28
Deficient	—	48	0.9[a]	15	25
			Females		
Control	—	69	1.9	0	0
	+	67	2.3	3	0
Deficient		81	3.2[a]	3	3

[a]Risk for tumor significantly increased. $P < 0.05$

Table 4 Hepatic content of choline and choline metabolites in procarbazine-treated rats

PCZ treatment mg/kg	wks	Diet	Hepatic content (% of control)[a] Choline	Phosphocholine
		Males		
300	5	Control	(149)	(210)
		Deficient[b]	1692	(220)
		Control, MTX[c]	344	(232)
		Females		
450	11	Control	(117)	(89)
		Deficient	160	(100)
		Control, MTX[c]	160	(72)

[a]Values in parentheses not significantly different from control not given PCZ
[b]Deficiency alone: hepatic choline 35%, phosphocholine 31% of control hepatic content
[c]Methotrexate

cause the patients' expected lifespans increases their risk of developing solid tumors.[46] There is a recent report of increased breast cancer risk in women treated at ages up to 30 years. For the first 15 years after therapy, the relative risk for breast cancer was increased to 6.3 in patients given both radiation therapy and chemotherapy: there was no demonstrable increase in risk for patients given either modality alone. After 15 years, the increased relative risk (14.8) was attributable to radiation therapy. Of the chemotherapeutic regimens used, only mechlorethamine, vincristine, procarbazine, prednisone, which includes PCZ, was associated with increased risk.[47]

These data, plus the calculation of similar carcinogenic potencies for several cancer chemotherapeutic agents in patients and rats,[48] emphasize the importance of further studies of dietary and nutritional influences on carcinogenicity of therapeutic agents in attempts to reduce the incidence of second tumors. Certain agents, including cyclophosphamide, which is a mammary gland carcinogen in rats, are used in combination therapy of breast cancer with MTX.[49,50] Consideration of methyl supply is of particular importance in such cases and also in cases in which total parenteral nutrition, which is low in choline,[51] is used. Use of MTX in long-term therapy of connective tissue diseases and other chronic, non-neoplastic disease also should be planned with consideration of dietary labile methyl intake and exposure to potentially carcinogenic agents.

Summary

In summary, dietary deficiency of lipotropic nutrients or labile methyl supply enhances "spontaneous" and chemical carcinogenesis in the liver of rats. The deficiency also enhances DMH carcinogenesis in the rat colon and PCZ carcinogenesis in the rat mammary gland. In the PCZ model, parallel studies using MTX to induce biochemical changes analogous in some characteristics to the lipotrope-deficient model also showed evidence suggestive of enhancement of carcinogenesis, although the results were not statistically significant. PCZ interfered with hepatic choline metabolism, particularly in lipotrope-deficient and MTX-treated rats. This effect may be related to the enhanced carcinogenicity of PCZ in the mammary gland.

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