Histol Histopathol (1995) 10: 457-462

Histology and Histopathology

Invited Review

Liver biochemical pathology of choline deficiency and of methyl group deficiency: a new orientation and assessment

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Summary. New information on the pathologic effects of a choline deficient diet in the rat, in relation to the biochemical events, has led to a new understanding and orientation of the pathogenesis of both acute and chronic consequences in the liver. The biochemical pathology of choline deficiency is quite different than that of methyl group (lipotrope) deficiency. These studies in our laboratory and elsewhere are generating new insights and hypotheses concerning the genesis of hepatocyte necrosis and hepatocellular carcinoma in the rat fed a choline deficient diet.

Key words: Choline deficiency, Fatty liver, Liver cell necrosis, Lipid peroxidation, Hepatocarcinogenesis

Introduction

The discovery by Best and Huntsman in 1932 of raw pancreas and its component lecithin and of the contained choline as the active ingredient in preventing and curing fatty liver in the depancreatized dog led to some of the most interesting studies in the pathobiology and pathogenesis of fatty liver, cirrhosis and liver cancer as well as new developments in the biochemistry of one carbon compounds, especially methyl groups.

A major uncertaintly concerning the possible role of feeding a choline-deficient (CD) diet in liver cancer occurred around 1960 when the contamination with aflatoxins of the dietary peanut meal used to make a CD diet and the carcinogenic effects of aflatoxins were discovered (e.g. Newberne et al., 1964, 1982; Busby and Wogan, 1984).

A second development occurred only a few years ago with the rediscovery that "pure" CD diet with methyl-group adequacy induced liver cell cancer in the absence of contamination with any known carcinogens (Mikol et al., 1983; Ghoshal and Farber, 1983, 1984; Yokoyama et al., 1985).

A third development was the discovery that the acute short-term liver changes seen with choline deficiency are quite different than those seen with lipotrope ("methyl group") deficiency (see Ghoshal and Farber, 1993). The major differences are presented in Table 1. The differences appear to be of a major magnitude, especially the apparent difference in the induction of hepatocellular carcinoma. So far, liver cell cancer induction with choline deficiency is well established while that with methyl group deficiency is uncertain.

This brief review will emphasize the "pure" CD syndromes, since the biochemical pathobiology of methyl groups (lipotrope) deficiency has been reviewed many times during the past several decades, even though the distinction between lipotrope deficiency and choline deficiency pathobiology was not made until very recently (Ghoshal and Farber, 1993).

Choline deficiency

Choline is a positively charged, quaternary ammonium compound and is the base in the phospho-lipid lecithin. Unlike other simple phospholipids, lecithin is universally present in every cellular membrane, both animal and plant. We obtain our major supply of choline not as free choline but by consuming lecithin. Due to the abundance of choline throughout our natural environment (Engel, 1943), the possibility of choline deficiency is not a common occurrence. Some workers mistakenly combined choline deficiency with methyl group deficiency.

It should be appreciated that as a methyl group donor, choline is weak and does not have any advantage over methionine, because only one methyl group is donatable. Also, choline must first be oxidized to betaine before it can act as a methyl donor for methionine synthesis. It has been shown that choline deficiency syndromes cannot be rectified fully and efficiently by methionine (Young et al., 1956). Choline appears to be important

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Table 1. Comparison of major differences between pathologic consequences in the rat with choline-devoid and lipotrope-deficient diets (Ghoshal and Farber, 1993).

CONSEQUENCE	CHOLINE-DEVOID	LIPOTROPE-DEFICIENT
1. Body weight	Good weight gain	Poor or no weight gain
2. Fatty liver	Very rapid periportal (zone 1)	Rapid central (zone 3)
3. Necrosis	Early (4.5 to 5 days) focal and widespread; at least 50% of hepatocytes within 2 weeks	None. Only some ill-defined hepatocyte injury associated with fatty cysts at a late period
4. "Fatty cysts" (lipodiastemata)	Not seen	Regularly seen after many weeks of LD diet
5. Cirrhosis	Very infrequent, even after 2 years	Very frequent, almost every rat
6. Hepatocellular carcinoma	Frequent - at least 50 to 70% of male rats by 2 years	Uncertain

mainly because of its structure and not because of its methyl groups alone. Choline deficiency disease is much more than methyl deficiency. In this respect the following observations should be considered: (a) a choline devoid diet which contains just adequate levels of methionine causes deficiency symptoms while methionine in blood and other tissues, including liver, remains at control levels (Sidransky et al., 1985); (b) it requires about 2 to 3 times more methionine than choline on a molar basis to counteract the effects of choline deficiency (unpublished observation). In choline deficiency, the SAM (S-adenosylmethionine) pathway increases by 2-3 times (Lombardi et al., 1969). The SAM pathway synthesizes lecithin in the membrane by transferring methyl groups to the ethanolamine in phosphatidyl ethanolamine. Lecithins synthesized by transferance of methyl groups to ethanolamine not only differ in the nature of their fatty acid content from the pre-existent lecithin synthesized by Kennedy pathway but are also not sufficient to handle the shortage of choline, although there is no shortage of methionine (methyl group). These results suggest that the pathobiology of choline deficiency is not a reflection of methyl group deficiency. Several years ago Hartroft (1973) came to a similar conclusion. His conclusion was that it is the nature of choline and not the methyl groups which are responsible for choline deficiency syndromes. He used arsenocholine (AS) instead of choline and found that replacement of nitrogen in choline by arsenic with retention of the 3 methyl groups changes the physiological role of the choline, but not of the methyl groups.

Acute and long term effects of choline deficiency

A diet devoid of choline when given to male Fischer 344 rats induces a highly reproducible series of changes in the liver (Ghoshal and Farber, 1984) (see Table 2).

Table 2. Sequence of known liver changes with dietary choline deficiency.

TIME	BIOCHEMICAL PATHOLOGY
6 to 8 hrs	Fatty liver, periportal (not central) progressively involving all the liver cells by days 4 to 5
24 hrs	Lipid peroxidation in nuclei
48 hrs	Alkali-sensitive alteration in DNA
72 hrs	Increase in activity of phospholipase A_2 in microsomes (but not nuclei)
4.5 to 5 days	Onset of progressive liver cell death with at least 50% by day 14
5 days	Lipid peroxidation in mitochondria
10 weeks	Initiation of liver carcinogenesis with appearance of rare resistant hepatocytes with resistance phenotype
1 year	First appearance of hepatocellular carcinoma with metastasis

Within 6-8 hours, the liver begins to accumulate triglycerides (triacylglycerols) in the hepatocytes in zone 1, spreading to zones 2 and 3 to involve the whole liver by days 4 to 5. Previously, it was considered that choline deficiency caused zone 3 (centrilobular) fat accumulation (Newberne et al., 1982). What was studied was lipotrope, not choline deficiency. Since choline has been linked to removal of triacylglycerols (triglyceride, "fat") from the liver, research became focused on the mechanism of this fat removal. In order to generate rapid and heavy deposition of fat in the liver, not only choline but also methionine, vitamin B12 and folic acid were removed from the diet. Many authors have designated this diet as CD. A more appropriate term is lipotrope deficient (Best et al., 1935; Young et al., 1956). This diet was not only nutritionally very inadequate but also had other complications. In other words, lipotrope deficiency and choline deficiency have radically different effects on the liver. These differences have been adequately discussed (Ghoshal and Farber, 1993).

The next temporal occurrence in the liver in choline deficiency is the appearance of lipid peroxidation in the nuclear membranes as detected by diene conjugates within 24 hours and by the appearance of aldehydes both histochemically and chemically (Rushmore et al., 1984, 1987; Ghazarian, 1993). This is followed by DNA alteration at 48 hours (Rushmore et al., 1986). Within 3 days, phospholipase A_2 increases in microsomes but not in nuclei (Kapoor et al., 1992). By day 5, one can detect mitochondrial lipid peroxidation. Hepatocyte cell death appears at 4 1/2 to 5 days. This increases so that about 50% of the liver shows cell death by 14 days (Ghoshal et al., 1983). Initiation of hepatocellular carcinogenesis, as assessed by the appearance of rare resistant hepatocytes with a special "resistance phenotype" (Roomi et al., 1985; Farber and Sarma, 1987; Ghoshal et al., 1987), becomes evident at about 10 weeks after beginning the exposure to the CD diet (Ghoshal et al., 1987). Hepatocellular carcinoma appears at about 1 year (Ghoshal and Farber, 1983, 1984; Mikol et al., 1983;

Yokoyama et al., 1985). This temporal sequence of events is shown in Table 2. The acute changes from the triacylglycerol accumulation to the appearance of resistant hepatocytes in the liver are very reproducible and appear in 100% of the rats. More than 50% of rats eventually develop liver cancer (Ghoshal and Farber, 1984). This CD model is very useful to study the different alterations in the liver including cell death.

Evidence of free radical involvement in choline deficiency

Lipid peroxidation in the nuclear membranes (both inner and outer) within 24 hours of CD diet feeding is the strongest evidence of free radical activity. This was supported not only by the subsequent appearance of aldehydes (Esterbauer, 1982) but also by the demonstrations that lipid peroxidation can be completely prevented by (a) dietary supplementation with a radicophil, AD_5 (Ghoshal et al., 1990), calcium and strontium (Ghoshal et al., 1987); and (b) spin traps like i) α-phenyl-tert-butylnitrone (PBN), and ii) tert-nitrosobutane (tNB). All these agents not only prevent peroxidation of the membrane but also prevent the further sequence of DNA damage, cell death and cell proliferation. The spin traps also prevent the PLA_2 increase (Ghazarian, 1993). However, none of these can prevent fat accumulation in the liver; only choline supplementation can prevent the triacylglycerol accumulation.

Possible role of agents mentioned above in the prevention of lipid peroxidation

AD_5 (N-p-metoxyphenylacetyldehydrosoalanine) is a radicophile (Viehe et al., 1985). This compound has been shown to be a free radical scavenger for superoxide anion and hydroxy radical (Buc-Calderon et al., 1987; Buc-Calderon and Roberfroid, 1988). It has been shown in our laboratory (Ghoshal et al., 1990) that AD_5 can inhibit in vivo-induced microsomal lipid peroxidation.

Calcium and strontium

How does excess Ca^{2+} protect the membrane from peroxidation? Normally, free radicals are believed to be generated in small amounts during oxidative reactions. Membranes have many defense mechanisms by which this small amount of free radicals can be counteracted. However, when excessive generation of free radicals or reduction of defense mechanism occur, this condition can become unbalanced. What is the mechanism by which choline deficiency can unbalance the system? In this respect choline could have a dual role. It has been shown by in vitro experiments (Miyazawa et al., 1984) that lecithin (choline) can breakdown preformed lipid hydroperoxides. Another property of choline is its positive charge. In choline deficiency, the reduction of choline in the membrane (Kapoor et al., 1992) may make the membrane relatively more negatively charged and possibly susceptible to free radical attack. Calcium has two positive charges. Calcium in moderate excess may attach itself to the membrane and presumably restore the positive charge. The addition of extra calcium to a CD diet prevents all the early CD-induced changes except the fat accumulation. This property of extra calcium is shared by strontium which has also two positive charges.

Spin traps

The role of spin traps on the prevention of free radical attack may be more straightforward. Spin traps are used for capturing and identifying free radicals. When rats were pretreated with spin traps like tNB and PBN before the CD diet was started, free radical formation, as measured by lipid peroxidation (Recknagel and Ghoshal, 1966a,b), was prevented. Pretreatment with spin trap agents prevented lipid peroxidation, DNA damage, PLA_2 activation, and cell death but not the accumultion of triacylglycerols.

Although there appears to be considerable evidence for the presence of free radicals in the livers of rats after CD exposure, the exact identity of the free radicals has yet to be determined.

A hypothesis relating CD to carcinogenesis

The ability of free radicals to attack and alter DNA has been shown by in vitro experiments (Slaga et al., 1981; Emerit and Cerutti, 1982; Rajalakshmi et al., 1982; Ames, 1983). It has been proposed that a diet devoid of choline without any added carcinogen initiates rat hepatocytes by free radicals generated in the nuclear membrane. As depicted in Figure 1, the temporal sequence of events could be: a) free radical generation, b) DNA damage, c) PLA2 activation, d) cell death, e) cell proliferation, f) initiation, and g) cancer. This suggested sequence for carcinogenesis with CD diet is modelled after a current hypothesis of liver cancer induction by chemical carcinogens (Fig. 2) (Farber and Sarma, 1987). Although the involvement of free radicals in the initiation process of carcinogenesis is suggested

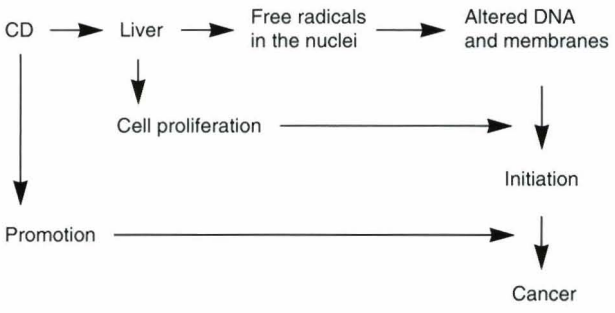

Fig. 1. Liver carcinogenesis with CD diet possible mechanism.

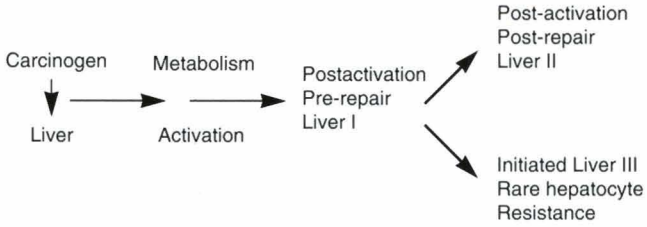

Fig. 2. Liver cancer induction (shown up to initiation) by chemical carcinogens.

by a reasonable data base, the data to implicate free radicals in the promotion and progression phases of carcino-genesis are at best only possibilities.

Cell death in choline deficiency and role of phospholipase A2

Phospholipase A_2 (PLA_2) removes arachidonic acid from the second position of glycerol of phospholipid of the membrane. The recent discovery that PLA_2 preferentially hydrolyzes peroxidized fatty acid esters in phospholipid membrane (Van Kujik et al., 1987) suggested the possible role of PLA_2 as a protector of membrane lipids from the injury due to lipid peroxidation.

Very recently a distinct role for PLA_2 in liver cell death due to choline deficiency has been shown (Ghazarian et al., 1994). It has been demonstrated (Table 1) that the induction of lipid peroxidation, DNA damage, excess of PLA_2 enzyme activity and cell death in the rat liver with a CD diet can all be inhibited by pretreatment with spin trap tert-nitrosobutane (tNB) (Ghazarian, 1993). When free radical generation and DNA damage is allowed to occur with the CD diet and then the increase in PLA_2 activity is inhibited by PBx, an oligomer (n=6) of prostaglandin B_1 (Franson and Rosenthal, 1989), cell death is also prevented (Ghazarian, 1993).

This suggests that even in the presence of excess free radicals and damaged DNA, PLA_2 induction in the liver is playing a role in cell death due to CD. In other words CD diet induces a free radical increase which generates liver cell death through a chain reaction in which PLA_2 activity is in the downstream.

How does exposure to a choline deficient diet result in excess free radicals?

There are at least two general possibilities:

(a) A CD diet may increase the rate of generation of free radicals such that their rate of production becomes more rapid than their rate of degradation or neutralization by the different antioxidant mechanisms in the living cell.

(b) A CD diet may decrease the ability of the hepatocyte, especially the membranes of the cell, to handle and destroy lipid peroxides and other products of free radical action. Under these conditions, the rate of degradation or neutralization of free radicals and/or their consequences become much less than the normal rate of generation.

Table 3. Evidence for free radical activity in liver with choline deficiency.

1. Lipid peroxidation in nuclei by 24 hours.
2. Radicophile AD_5 (N-p-methoxyphenylacetyldehydroso-alanine) prevents lipid peroxidation.
3. Free radical trapping agents, such as a-phenyl-tert-butylnitrone (PBN) and tert-nitrosobutane (tNB) ( ) prevent lipid peroxidation.
4. Calcium and stronium (Goshal et al., 1987) prevent lipid peroxidation.
5. Lipid peroxidation is followed in time, by DNA alteration and hapatocyte cell death and all of these effects are prevented by the above agents..
6. Inhibition of phospholipase A_2 (PLA_2) increase lipid peroxidation, that now appears also in microsomes
7. Aldehydes appear in liver cells following lipid peroxidation as with other instances of lipid peroxidation in the liver (e.g. see Esterbauer, 1982) and these are prevented by PLA_2 as well as by inhibiting lipid peroxidation.
8. Radicophile AD_5, PBN and tNB prevent not only lipid peroxidation but also DNA alterations and cell death even when administered after lipid peroxidation appears in the nuclei.

There is no readily evident mechanism for pursuing the first hypothesis. No data are known by these authors that could support such an hypothesis.

There are, however, data which offer some support, consistent with the second hypothesis. The dietary exposure to a CD diet rapidly leads to major changes in the phospholipid composition of the hepatocyte membranes. These changes are of a considerable magnitude and could theoretically interfere with the ability of the cell to exert its antioxidant-anti-lipid peroxide repair systems. Although the evidence is by no means conclusive, it does offer an orientation that could be exploited for subjecting the hypothesis to increasingly rigorous critical experiments. The biological consequences of a choline deficiency are sufficiently important and impressive to warrant such an approach

General comments

1) Choline as a base of phospholipid lecithin is available abundantly throughout the vegetable and animal world with the possible exception of some parts of the world where malnutrition or where people are eating spoiled grains. Thus, it is most likely that choline deficiency in humans is quite uncommon.

2) It should be realized that choline deficiency is not methyl deficiency. Although choline has 3 methyl groups, only one is donatable, after oxidation to betaine. Choline deficiency syndrome cannot be rectified fully and efficiently by methionine.

3) One cannot consider choline deficiency and lipotrope (choline, methionine, B_{12} and folic acid) deficiencies as the same. The syndromes are quite different.

4) The occurrence of cancer by eliminating one dietary component without the addition of any carcinogen is a unique animal model in which to study step by step not only liver carcinogenesis but also in vivo free radical generation and liver cell death without the interference of an xenobiotic.

Acknowledgements. We would like to express our sincere thanks to Lori Cutler for her excellent secretarial help. The work from this laboratory which has been presented in this article was supported in part by grants from the US Public Health Service (CA 41547 and CA 21157) and from the National Cancer Institute of canada, and the Medical Research Council of Canada.

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