Review

Tumorigenesis, protooncogene activation, and other gene abnormalities in methyl deficiency

Benito Lombardi and Martin L. Smith

University of Pittsburgh School of Medicine, Department of Pathology, Pittsburgh, PA USA; and Pittsburgh Cancer Institute, Pittsburgh, PA USA

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Introduction

The methyl deficiency model of hepatocarcinogenesis is notable because hepatocellular carcinomas (HCCs) arise in rodents chronically fed methyl-deficient diets, without intentional or adventitious exposure of the animals to chemical carcinogens,[1-5] or genetic manipulations of the animals.[6-9] The tumorigenicity of these diets, therefore, appears to reside solely on effects they have on rodent liver. Unfortunately, a large number and variety of those effects are known, and have been known for some time,[10-17] while newly discovered ones are being steadily reported in the literature. This situation obviously complicates the evaluation of and search for which effect(s) contribute(s) to the tumorigenicity of the diets, and, in particular, directly results in, or leads to, the genomic alterations that are responsible for cancer induction. Nonetheless, such an effort is ongoing in several laboratories, and several promising leads and working hypotheses are being pursued at the present time.

In this paper we shall review briefly the results obtained by us in this effort, as well as the hypothesis that has been underlying our work. Our studies have been performed on rats fed semi-purified diets, either adequate in choline (CS diet), or essentially devoid of choline (CD diet), both providing about 50% of the methionine daily requirement of young, growing rats. Their detailed composition has been previously reported.[4,18] When fed initially to young male Fischer-344 rats, the CD diet has been shown to induce HCCs consistently, with incidences varying from 30% to 70%, depending on experimental modalities, and, especially, the duration of the treatment.[3,5,19] These incidences are comparable with those observed in the same strain and sex of rats fed other formulations of methyl-deficient diets,[2,20] with one exception.[19]

The tumorigenicity of methyl-deficient diets: a working hypothesis

In male Fischer-344 rats, the CD diet causes a condition of hepatitis that results from repeating cycles of liver cell death and regeneration, lasting for as long as the diet is fed.[21-26] Induction of such a hepatitis, though, is not unique to the CD diet, but appears to be a common effect of all methyl-deficient diets, and to be actually much more accentuated by some of them.[19,20] We attach a great significance to this hepatitis and the mitogenesis[27] that causes it because we think that it is the major single effect that underlies the hepatocarcinogenicity of methyl-deficient diets (*Figure 1*). Female Fischer-344 rats are largely resistant to CD diet-induced mitogenesis, and do not develop HCCs.[28,29]

The role that mitogenesis may have in the pathogenesis of human and experimental tumors is the object of much consideration and discussion at the present time, and the many ways in which it could lead to genomic alterations, relevant to cancer genesis, have been considered extensively.[27,30,31] Two broad classes of mechanisms may be envisaged, one of "accidents" occurring during the processes of cell replication and the other of events related instead to cell death, such as the inflammatory response elicited by the need to remove cell

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Address reprint requests to Dr. Benito Lombardi at the University of Pittsburgh School of Medicine, Department of Pathology, Room 711, Scaife Hall, Pittsburgh, PA 15261, USA.
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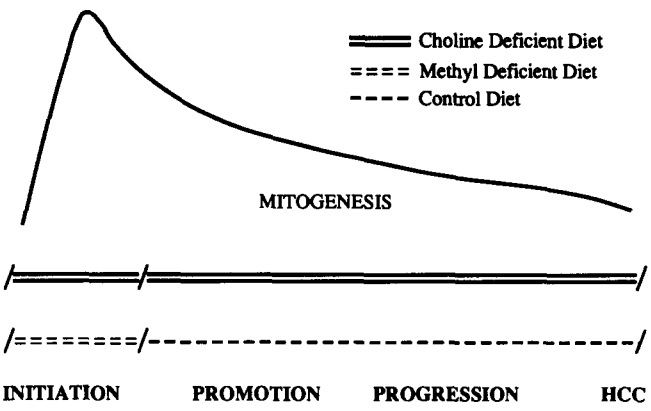

Figure 1 Time-course of the intensity of mitogenesis (cell proliferation induced by cell death; solid line) in relation to the initiation, promotion, and progression stages of hepatocarcinogenesis in male Fischer-344 rats fed the CD diet throughout (double solid lines[25,26]). In two separate studies,[26,97] HCCs developed in male Fischer-344 rats fed a methyl-deficient diet for 3 to 4 months (double dotted lines), followed by feeding control diets (single dotted line), indicating that initiation occurs during the most intense period of mitogenesis.

debris. Cancer genesis is a multistep process,[32,33] in which transition of one step to the next is determined by the occurrence of genomic alterations that generate new clones of cells, having heritable phenotypic changes that favor the genesis of yet further altered clones.[34,35] The mitogenesis caused by methyl-deficient diets in the liver, a normally mitotically quiescent organ, could very well generate such relevant genomic alterations directly as the result of "accidents" in cell replication processes. On the other hand, the enhanced rate of cell division could lead to the "fixation" of other types of liver cell DNA damage or of epigenetic changes, such as those that could arise from nucleotide pool imbalances,[36] hypomethylation of genes,[37-40] or from the action of oxygen free radicals produced by inflammatory cells. Another DNA change that can probably be ascribed to the inflammatory response that accompanies mitogenesis by the CD diet is formation of significant levels of 8-OHdG,[41,42] which is mutagenic.[43]

The neoplasia stage

In approaching the study of proto-oncogene activation by the CD diet, we have so far focused mainly on the very last stage of the model, because the approach we adopted was to first screen tumors for the presence of relevant alterations, and for those detected, to endeavor to establish then whether they could have occurred at earlier times in the treatment of the animals. One criterion we used in assessing the relevance of any alteration was that it be present not only in the tumors, but also in nontumoral portions of tumor-bearing livers, or in nontumor-bearing livers of similarly treated rats; that is, to have an indication that the alteration might have occurred before, and might have thus contributed to, the genesis of the tumors.

The level of expression of several proto-oncogenes was examined, including *H-ras, K-ras,* and *N-ras,*[44] and *myb, n-myc, erb-b,* and *raf* (unpublished observations). Various degrees of increase in the transcript level of these proto-oncogenes, relative to those in the liver of rats fed the control CS diet, were observed in tumors, but not in nontumoral portions of tumor-bearing livers, or nontumor-bearing livers of rats fed the CD diet for an equal period of time. Southern blot analyses revealed no structural alteration of the genes, such as translocation, deletion, or amplification. Moreover, in unpublished studies in collaboration with S. Sukumar, 3T3 cell transformation assays performed on 10 HCCs showed an absence of transforming genes, effectively ruling out the presence of *ras* gene mutations. The gene transcript elevations were thought therefore to be mostly a reflection of active cell proliferation in the tumors, rather than contributors to their genesis, and were considered thus as not "relevant."

The opposite conclusion was, however, reached in studies concerning the *c-myc* proto-oncogene.[45] Fourteen of 14 HCCs analyzed were found to carry an amplification of this gene, ranging from two to 70 fold, and were accompanied by corresponding increases in transcript levels. The amplification was larger in tumors that arose in rats fed sequentially the CD and control CS diets than in tumors that developed in rats fed the CD diet throughout. In the former, low levels of *c-myc* amplification were observed in nontumoral portions of tumor-bearing livers, and amplification of the gene was present also in 2 HCCs that developed in rats fed the CD diet for only 3 months, followed by feeding the control diet for 13 months. Thus, either the amplification itself occurred early, or the stage for its later occurrence was set early in the treatment of the rats. It appears, therefore, that occurrence of *c-myc* amplification might be one of the genomic alterations contributing to the neoplastic transformation of cells in this nutritional model of hepatocarcinogenesis.

More recently, we directed our attention to two tumor suppressor genes, the *RB* and *p53* genes. By Southern blot analysis, a structural alteration of the *RB* gene was detected in two of 13 HCCs analyzed, and in none of 13 non-tumoral portions of tumor-bearing livers. The alteration consisted of novel hybridization bands, which were unique to individual tumors.[46] Alterations of the *RB* gene, therefore, do not appear to be one of the more prominent features of CD diet-induced HCCs. In contrast, mutations of the *p53* gene were found to be present with high frequency in these HCCs.[47] Availability of highly specific antibodies to mutant *p53* proteins made it possible to study this alteration not only by cDNA sequencing, but also by immunoblotting and immunostaining. Twenty-two of 27 tumors examined by immunostaining and 18 of 20 analyzed by immunoblotting revealed the presence of mutant *p53* proteins (*Figure 2*). cDNA sequencing was performed on 11 HCCs that expressed mutant *p53* proteins. Seven were found to contain point mutations within the 120-290 codon region of the gene, and one a microdeletion in the same region. The site of the mutations was unique in each tumor, because it involved either different codons or different bases in the same codon; no mutational

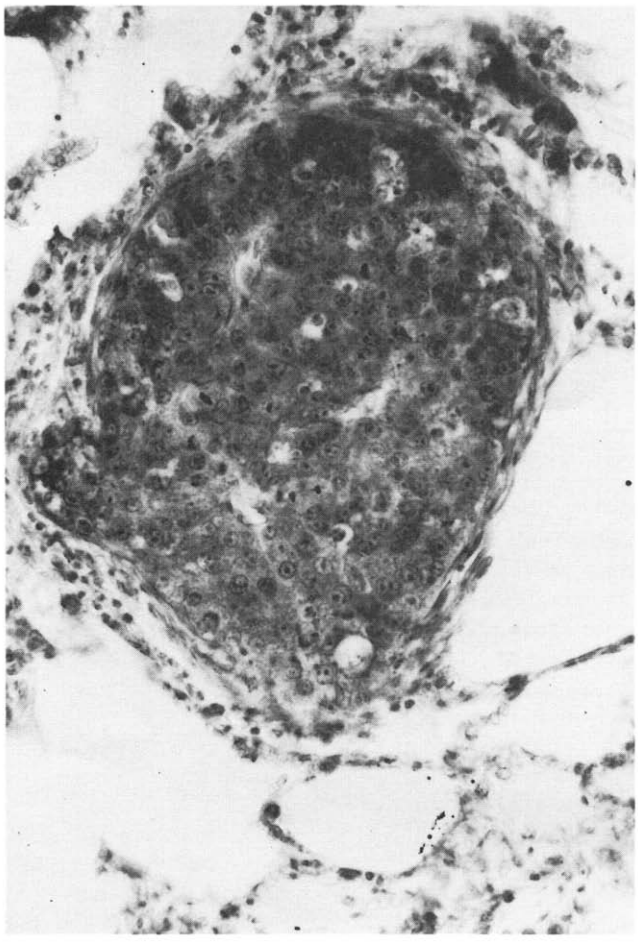

Figure 2 HCC metastasis in the lung of a male Fischer-344 rat fed the CD diet for 6 months, and then the CS diet for 10 months. The section was immunostained with the anti-p53 monoclonal antibody PAb240 and counterstained with hematoxylin. Note the heavy presence of reaction product (black), predominantly in the cell cytoplasm.

hot spot was therefore observed. These findings are consistent with the lack of evidence that the diet fed to the rats contains aflatoxin B_1, or that the animals are exposed during treatment to other relevant chemical carcinogen contaminants in their total environment.[2,3,48–50] On the other hand, mutation and amplification are structural gene alterations that could very well originate from a cell replication "accident,"[30,51] and the observed randomness of the *p53* mutations is also consistent with such a notion.

p53 and *c-myc* are both involved in the regulation of cell proliferation,[52,53] and it may be more than just a coincidence to find that they are both altered in HCCs arising after a protracted period of highly abnormal liver cell turnover. In this respect, however, it should be noted that alterations of neither the *p53* nor the *c-myc* genes have been detected in HCCs that arise in transgenic mice carrying the hepatitis B virus (HBV) surface antigen.[54] This model of hepatocarcinogenesis shares with the CD-diet model the fact that the tumors also develop after a protracted period of severe liver injury and repair, also considered to be the primary factor in the pathogenesis of these tumors.[55] Reactivation of the insulin-like growth factor II gene is one gene change observed in HBV transgenic mice.[56] By contrast, we found that expression of this gene is unchanged throughout the stages of CD-diet hepatocarcinogenesis, including the tumors (*Figure 3*). Distinct differences appear to exist in the set of oncogenes that contribute to hepatocarcinogenesis in different animal species.[57–60] The discordant findings made in the two models, therefore, may reflect species differences more than being contradictory of the primary pathogenetic role of mitogenesis. In human HCCs, *c-myc* alterations[61–67] and *p53* mutations[68–80] have been reported to occur with moderate frequencies. The type of *p53* mutations has been also observed to vary in different regions of the world.

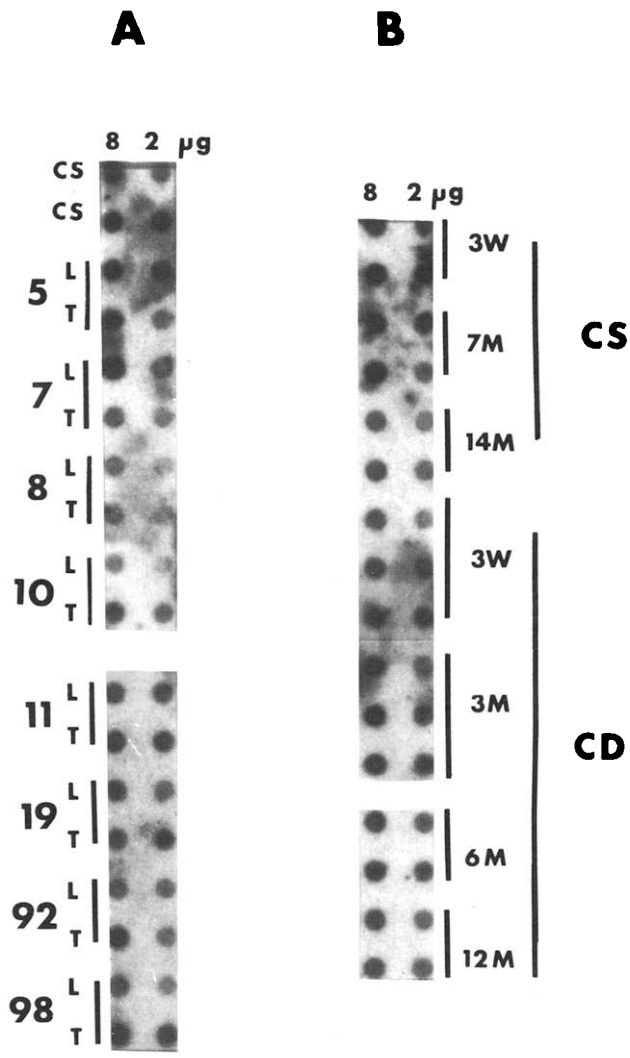

Figure 3 Dot-blot analyses of 8 and 2 µg of total RNA, probed with an IGF-II cDNA. (A) CD-diet-induced HCCs (T), nontumoral portions of tumor-bearing livers (L), and livers of rats fed the control diet (CS); no appreciable differences are apparent. (B) Time course expression of IGF-II in the liver of rats fed exclusively either the CD diet, or the control diet, for the number of weeks (W) or months (M), as indicated; again no appreciable differences are seen.

In areas in which both aflatoxin B_1 and HBV are risk factors, the mutations have been found to be prevalently clustered around codon 249 of the gene, pointing to aflatoxin as the most likely causative agent of the tumors.[68,70] On the other hand, in areas where HBV but not aflatoxin B_1 is a risk factor, the mutations have been found to be scattered throughout the *p53* gene, implicating the abnormal cell turnover due to the HBV hepatitis as being most likely responsible for the mutations and the genesis of the tumors.[74] The CD diet model of hepatocarcinogenesis in the rat, therefore, seems to mimic fairly well the latter type of human HCCs.

The progression and promotion stages

In rats fed the CD diet, immunochemical evidence was also obtained that shows that the occurrence of *p53* mutations precedes tumor development, at least at a time somewhat late in the natural history of these tumors. Positive immunoblotting and/or immunostaining results were obtained in nontumoral portions of tumor-bearing livers, and in nontumor-bearing livers of rats fed the CD diet for 10 months or longer.[47] In the latter, focal areas of mutant *p53* positive hepatocytes were seen, which, in a few cases, could be clearly identified as preneoplastic nodules. However, the majority of the positive areas had a cytology and overall morphology more consistent with those of regenerative nodules in a fibrotic liver. This observation obviously deserves further scrutiny and study, because it could provide clues about the cell lineage in the development of HCCs in this model. It is indeed not clear to us, at the present time, what this cell lineage is. In particular, we have as yet no direct evidence of the early presence in the liver of focal clones of cells bearing *p53* mutations, *c-myc* amplification, or alterations of any other proto-oncogene.

Other groups have reported the early occurrence in rats fed methyl-deficient diets of epigenetic changes of great potential significance and relevance to the tumorigenicity of these diets. Those involving gene methylation are reviewed in this issue by Christman,[81] while Shinozuka reviews changes in the expression of growth factors and their receptors,[82] and Dr. S.H. Zeisel reviews changes in signal transduction pathways.[83] There appears to be little doubt that epigenetic changes can play a critical role in a carcinogenic process, as in the case of those that favor the expansion of clones of altered cells.[84] To be relevant and significant, though, it seems that an epigenetic change would have to facilitate or directly lead to the generation of a new clone of cells at greater risk of evolution to cancer, if sequential clonogenicity of newer "hits" is indeed the only means of cancer genesis.[85]

The initiation stage

The earliest clones of altered cells that have been seen so far in the liver of rats fed methyl-deficient diets are foci of γ-glutamyltranspeptidase (GGT) or glutathione S-transferase, placental form (GST-P) positive hepatocytes,[4,20,86–88] the same type of early lesion that is almost invariably seen in chemical models of hepatocarcinogenesis.[89] In the latter, the foci represent clonal expansion of individual cells initiated by the chemical carcinogens.[90] Because chemical carcinogens are not involved in the tumorigenicity of methyl-deficient diets, two primary, but not mutually exclusive, possibilities therefore exist, insofar as the stage of initiation is concerned: either the diets act as complete carcinogens, able to initiate liver cells de novo, as well as to promote the evolution of these cells to cancer; or the diets act simply as promoters of the evolution to cancer of liver endogenous initiated cells (EICs).

The existence of EICs in rodent liver,[91] including the liver of Fischer-344 rats, is now supported by a fairly large body of experimental evidence.[4,89] Two specific traits are characteristic of these cells: (1) they bear some of the same phenotypic changes that are used to identify and characterize evolving cells initiated by chemical carcinogens, such as GGT or GST-P positivity; and (2) they are present either as single cells or clusters of various sizes in the liver of colony animals or of control rodents not treated with chemical carcinogens. EICs have been seen in the livers of young and untreated male Fischer-344 rats,[92] and in unpublished observations we made in collaboration with Shinozuka, in the livers of newborn male Fischer rats.

A possible, if not probable, involvement of EICs in the tumorigenicity of methyl-deficient diets, was clearly indicated by the results of short-term studies recently performed by Shinozuka et al.[88] Upon feeding a CD diet to young male Fischer-344 rats not exposed to a chemical carcinogen, an increasing number of single GST-P–positive cells were observed to appear in the liver, which gradually converted into doublets, triplets, and eventually into foci of GST-P–positive hepatocytes. Thus, to the extent that GST-P is indeed a marker of initiated cells, and that hepatocytes in GST-P positive foci are indeed precursors of HCCs, these investigators concluded that a CD diet could very well act as a pure promoter of the clonal expansion and evolution of EICs to HCCs.[88] Observations consistent with a participation of EICs in the tumorigenicity of methyl-deficient diets have been made also in long-term studies. At least two groups have noted that only a very limited number of focal lesions develop before the HCCs, a number comparable with that with which spontaneous foci are observed to occur in the livers of colony rats.[20,26] Occurrence of EICs, moreover, is not exclusive to the liver.[4] In experiments of 16-month duration or longer, we have observed the development of pre-neoplastic, benign, and occasional malignant lesions in the pancreas of male and in the pancreas and breast of female Fischer-344 rats. These lesions, however, developed with similar incidences irrespective of whether the rats were fed the CD diet, or the control, CS diet.[29,93] We interpreted therefore their development as being simply the result of the evolution of EICs present in those organs. The arguments presented above, of course, do not amount to

proof that methyl-deficient diets act merely and solely as promoters. However, it seems to us that such a possibility, and the whole phenomenology and relevance of EICs to carcinogenesis processes, cannot be simply ignored as proposed by Farber.[94] In this respect, one is reminded of the views long held by this author vis-a-vis the concept of a stem cell compartment in rodent and human livers. It is amazing indeed to see the evolution undergone by this concept,[95,96] since investigators decided to look into it, rather than to dismiss it.

Unfortunately, a clear and convincing proof that the diets act as complete carcinogens is also not available as yet, and the list of potential mechanisms whereby these diets could lead to an initiation of liver cells de novo is certainly not short.[4,15-17,37] In recent years, an attractive hypothesis has been formulated by Ghoshal et al.,[97] at least insofar as the CD diet is concerned. Briefly stated, the hypothesis consists of generation by this diet of free radicals in the liver that would trigger a peroxidation of nuclear membrane phospholipids, leading to DNA damage, and, thereby, to an initiation of liver cells de novo.[88] It is surprising, though, that in almost a decade of work on this hypothesis, Ghoshal and co-workers have produced as yet no direct evidence of the presence of conjugated dienes in the phospholipids of liver nuclear membranes. When this critical question was examined by us, none was found.[98] Moreover, the origin of the conjugated dienes that are detected in the total and neutral lipids of these livers was traced to stable fatty acids with conjugated dienes present in the hydrogenated fat used in the formulation of the CD diet.[99] Indeed, when diets containing only corn oil were used, neither we[98,99] nor Ghoshal et al.[100] could detect conjugated dienes any longer in any of the liver lipids. Recently, another group, using an altogether different methodology, also found no evidence of liver phospholipids peroxidation in mice fed the same CD diet, while some of the mice nonetheless developed HCCs.[101] Relevant DNA damage, however, could result from the action of oxygen free radicals produced by inflammatory cells,[102,103] DNA modifications generated by altered metabolism such as imbalances in nucleotide pools[36] or formation of 8-OHdG,[41,42] or the epi-mutagenicity of gene undermethylation.[104] There is evidence that cells having the potential to evolve fully to HCC are already present in male Fischer-344 rats after the initial 3 to 4 months of feeding a methyl-deficient diet.[26,105] This is the time during which mitogenesis by the CD diet is the most intense (*Figure 1*).[15] Thus, initiation of liver cells de novo could also be a direct result of mitogenesis.[30] Finally, irrespective of the mechanism and nature of the first relevant event induced by these diets, EICs, as well as non-initiated hepatocytes, could be its targets. There are no obvious reasons why EICs would be spared the effects of these diets, including the first relevant event; as a result of it, though, they would be one step ahead of hepatocytes initiated de novo.[5,106] Methyl-deficient diets, therefore, could very well act at the same time as complete carcinogens and pure promoters; given the clonal origin of tumors,[107] it may be eventually feasible to test this possibility.

The mitogenesis of methyl-deficient diets

As acknowledged recently by Ghoshal and Farber,[94] Hartroft was one of the most careful pathologists to study liver changes in choline deficiency.[12] Here is his description of how far hepatocytes can accommodate the uniquely large[4,11] amounts of fat they have to contend with when rats are fed a CD diet: "If the animals are maintained on a low-choline diet for 1 or 2 months, many of the intracellular fat droplets are released from their parent cells by a process of rupture. The fat, now extracellular, is contained within the lumen of a cyst."[108] This description applies equally well, if not better, to the fatty liver induced by the CD diet currently used in both Ghoshal and Farber[24] (see *Figure 2,* taken after 2 weeks of feeding) and our laboratory (see *Figure 7,* taken after 4 weeks).[109] In light of present knowledge, it would be hard to conceive that hepatocytes can survive a rupture of the plasma membrane and remain viable. The mechanism described by Hartroft, therefore, might very well not be the only cause of cell death, and hence proliferation, in these livers, even though none other has been as clearly identified and documented as yet; however, it is certainly one major, if not the major, cause of it, as even Ghoshal and Farber appear to concede in their earlier paper.[24] If one considers that the rate of fat accumulation in individual hepatocytes is asynchronous, it is not surprising at all that such a mechanism can result in cell death and proliferation within a few days of feeding.

We think, finally, that the historical records show that the discovery of the lipotropic action of choline by Best[10] was not accidental,[94] but the result of a decade of persistent and very methodical work.[110]

Conclusions

In conclusion, considerable progress has been made in recent years in the study of the multi-faceted and complex effects of methyl-deficient diets on rat liver, and in the assessment of their potential relevance to the tumorigenicity of the diets. The overall picture, though, is still fragmentary, and much of the progress seems to have been made more at a phenomenological, rather than mechanistic, level. This appears to be particularly true in the case of the activation of whatever oncogenes, and the genesis and nature of the genomic alterations, that are required for the initiation, promotion, and progression of the HCCs induced by these diets.

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