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Chemical Liver Carcinogenesis: Monitoring of the Process by Flow Cytometric DNA Measurements

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The carcinogenic process in the liver was monitored from early nonneoplastic changes to the development of tumors in two well-known models; IP administration of dimethylnitrosamine (DMN) to newborn mice, and feeding of rats with acetylaminofluorene (AAF). Liver parenchyma cells and liver tumor cells were isolated by collagenase treatment and prepared for flow cytometric (FCM) measurements of nuclear DNA content. The changes observed were correlated to morphology and ^{3}H-thymidine incorporation.

The early preneoplastic changes in hepatocytes from DMN-treated mice were shifts in the DNA content to classes of higher nuclear ploidies. AAF-feeding of rats caused gradual distortion of the DNA content of nuclei from parenchymal cells. After 16 weeks of exposure, before any tumors were seen, a majority of the nuclei displayed an aneuploid DNA content.

The significance of these changes in the carcinogenic process is discussed, and a possible use of these models for detection of hepatotropic agents and agents causing aneuploidy (clastogens and turbagens) is proposed. The liver neoplasias (adenomas and hepatocellular carcinomas) induced by these models contained tumor stemlines with diploid DNA content, and some hepatocellular carcinomas showed aneuploidy. Nodules of hyperplasia contained diploid and tetraploid stemline.

Introduction

Flow cytometry has improved our ability to measure constituents of individual cells in a rapid and exact manner. Applied as a cell kinetic method, it quantitates the relative distribution of cells with G_1-S and G_2 phase DNA content (Fig. 1). In addition, flow cytometric DNA measurements can detect conditions like polyploidy, aneuploidy and clonal growth. These are biological phenomena which earlier methods like chromosome counting and microspectrofluorometry have shown to be associated with some aspects of carcinogenesis and tumor biology.

Polyploidy implies the multiple of chromosome number and DNA content of nuclei and is thought to accompany adaptation to extreme growth conditions. Polyploidy is linked to carcinogenesis, both as a finding in early stages of carcinogenesis and as an effect of carcinogens in several systems. The frequent occurrence of tetraploid or near tetraploid tumor stemlines also indicates that a polyploidization may take place at some stage in tumor development.

Aneuploidy may be caused by clastogens and turbagens. Clastogens are substances that cause chromosomal breaks, and turbagens interfere with the spindle apparatus. This can cause loss of DNA material or unequal distribution of DNA to daughter cells during mitosis. Agents causing aneuplody are strongly suspected to be carcinogens or teratogens, which can be detected by the micronucleus test, sister chromatid exchange and other cytogenetic methods as well as flow cytometry.

Clonal growth is a characteristic feature of tumors, and aneuploid DNA content of tumor stemlines is recognized as a marker of malignant growth, however euploid stemlines are also found both in malignant and benign tumors.

The occurrence of polyploidy, aneuploidy and clonal growth in liver carcinogenesis can be monitored by FCM as shown in the experiments summarized here. The role of these phenomena in the pathogenesis of liver tumors, and a possible use of these experimental models for testing of suspected carcinogens and cocarcinogens are discussed.

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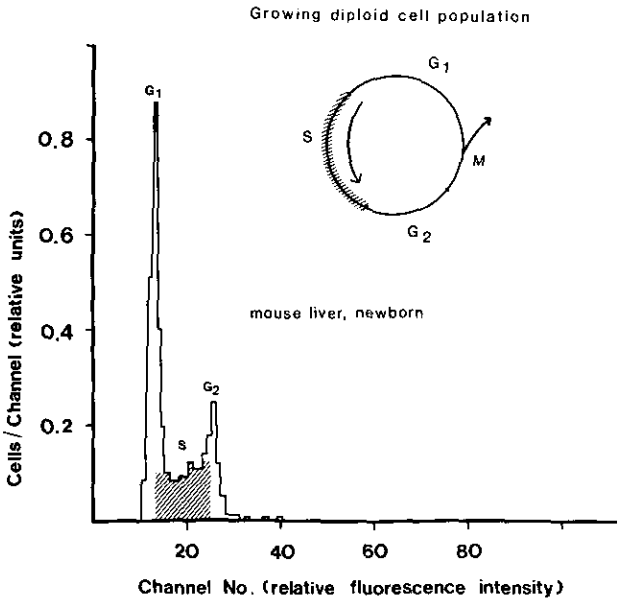

FIGURE 1. Relative distribution within the cell cycle phases (above) of hepatocytes from newborn mouse expressed according to DNA content in the FCM histogram (below). The diploid G_1 channel corresponds to DNA fluorescence of standard spleen lymphocytes.

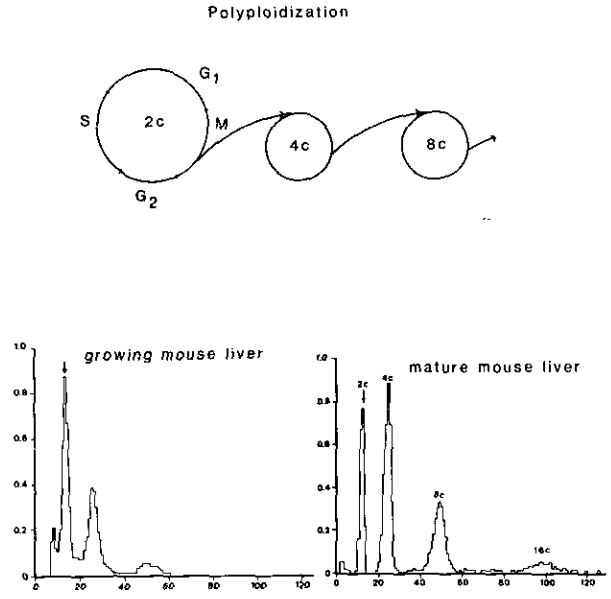

FIGURE 2. (Top) Polyploidization as cell cycles without mitotic division, thereby forming classes of higher ploidies. The histograms (bottom) show the DNA content of hepatocytes from growing mouse liver distributed in a diploid, a tetraploid and a small octoploid population, with S-phase pulses in between, and the ploidy classes of the mature mouse liver.

Material and Methods

Animal Models

The models used were the exposure of newborn mice to dimethylnitrosamine (DMN), and low-dose feeding of rats with acetylaminofluorene (AAF). In both series, the changes occurring during the treatment and the resulting liver tumors were investigated by flow cytometric measurements (FCM).

FCM shows that mature hepatocytes have the following distribution in the mouse liver: a few diploid cells, mostly tetraploid and octoploid cells and a few 16-ploid cells (Fig. 2) (*1*).

Rat hepatocytes comprise diploid, tetraploid and a few octoploid cells (Fig. 3). These differences in the distribution of hepatocytes in mice compared to rats, make it easier to detect shifts among the

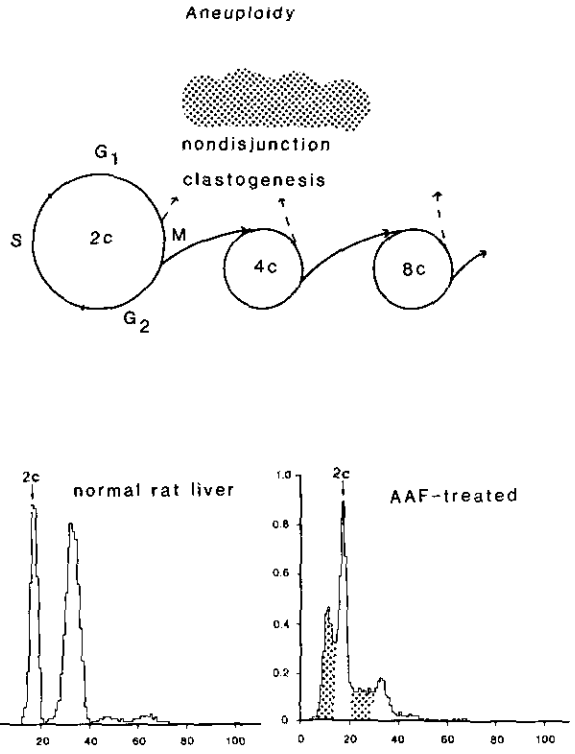

FIGURE 3. A proposed mechanism for the formation of aneuploid hepatocytes is shown (top). If substances interfere with the spindle apparatus causing nondisjunction or induce chromosome breaks with subsequent unequal mitotic distribution of genetic material or loss of genetic material, this will lead to gradually increasing populations with aneuploid DNA content. The speed of this process depends on mitotic activity and survival of altered cells. The histograms of normal rat hepatocytes (left) and hepatocytes from a rat exposed to AAF for 4 months (right) are shown (Digernes et al. manuscript in preparation). The shaded areas represent aneuploid DNA values.

ploidy classes in mice, especially because the admixture of nonparenchymal cells interferes very little with the results.

The normal process of polyploidization in mouse liver and its modulation by DMN given during a limited period was measured by FCM (2). The treatment schedule as described by Vesselinovich (3) consisted of 6 IP injections of a 0.4% solution of DMN in 0.9% saline (2 μg/g body weight) at 3-day intervals from the age of 6 days to the age of 21 days. A total of 54 mice (NMRI) of both sexes were exposed to this treatment, while 52 untreated mice served as controls. From each group, four animals (two of each sex) were killed at weekly intervals until the age of 52 days, when a stable ploidy pattern was reached.

Thirty treated animals and 24 control animals were kept until the age of 8 to 11 months, when most of the male mice and some of the female mice had developed liver tumors. These tumors were the source of the investigation on the ploidy of tumor stemlines, which were correlated with the morphological classification of the tumors as hyperplastic nodules, adenomas and hepatocellular carcinomas (4).

The rats were given low doses of AAF to try to avoid acute cell toxicity. Twenty-five male Wistar rats (180 g body weight) were given 0.025% AAF in a low-protein diet. From week 5 to the week 16 the animals were killed off at approximately weekly intervals. Samples of liver tissue were prepared for histology, and suspensions of viable hepatocytes were isolated by collagenase perfusion and centrifugation. The proliferative activity of these hepatocytes could then be examined by measuring thymidine incorporation in short-term cultures, and the DNA content of individual cells was measured by FCM (5).

This *in vivo/in vitro* approach has also been used for investigation of the proliferative response after hepatectomy, where a good correlation between amount of S-phase cells detected by FCM and thymidine labeling was found (5).

After the AAF treatment, we gave one group of rats 0.05% phenobarbital in their drinking water in order to promote the appearance of liver tumors. The animals were killed after 12-18 months. The tumors were examined histologically, and the tumor cells were analyzed by FCM.

Isolation of Hepatocytes and Tumor Cells

Mouse liver cells were isolated by short *in situ* perfusion through the portal vein with Ca-free collagenase solution, followed by incubation of liver tissue slices in the collagenase solution with Ca added after 30 min. After 25 min the slices were dispersed into a single cell suspension. The proportion of parenchymal cells was enriched to about 90% by two subsequent low-speed centrifugations.

Viable rat hepatocytes from the AAF-exposed rats were obtained by *in vitro* collagenase perfusion and low-speed centrifugation (6). Samples of this cell suspension were taken for FCM, and for *in vitro* incorporation of ^{3}H-thymidine in short-term cultures.

Visible liver tumors were isolated. One half of each tumor was dissected free from the surrounding liver tissue, sliced and dispersed into a single cell suspension by incubation in collagenase solution. The other half with surrounding liver tissue was fixed in formalin and processed for histological examination.

Preparation and Staining of Cells and Flow Cytometric DNA Measurements

Nuclei were prepared by cell lysis in distilled water and incubation with RNase (1mg/mL, 37°C, 60 min), followed by treatment with detergent (0.2%, 20°C, 30 min) and pepsin (0.4%, 37°C, 15 min). The nuclei were then stained with ethidium bromide (10 μg/mL in 0.1 *M* Tris buffer pH 7.5). Optimal staining was obtained after 15 min at 20°C.

Some samples were stained with mithramycin (25 μg/mL in 20% ethanol, 5 *mM* $MgCl_2$). The nuclei were prepared as described above but the RNase treatment was omitted. Optimal staining was obtained after 20 hr at 20°C.

The distribution of DNA fluorescence of the nuclei was determined by using an ICP 11 pulse cytometer. Each histogram represents measurement of about 5-10 × 10^3 nuclei. The preparation method described is shown to separate nuclei from binucleated hepatocytes only to a small extent (1). Such "pairs of nuclei" give only one pulse and are thus measured as a single nucleus. The ploidy classes of hepatocytes as quantitated by this FCM method therefore correspond largely to cellular ploidy.

Results and Discussion

Polyploidization of Mouse Hepatocytes and Effects of Dimethyl Nitrosamine

Maturation of the mouse liver was characterized by the development of polyploidy. During the first 6 weeks after birth the cell population changed from a purely diploid one to a combination of diploid, tetraploid, octoploid and 16-ploid cells (Fig. 2), and this distribution was normally maintained during the whole life span of the animal. The administration of DMN to newborn mice during the period when the polyploidization starts enhanced this process, and

the resulting shift to higher ploidies also involved the occurrence of 32-ploid nuclei. This state of increased polyploidy seemed to be maintained throughout adult life and was seen in both male mice, which are strongly susceptible to the hepatocarcinogenic effect of DMN, and in the less susceptible female mice. The increase in ploidy implied an increase in cellular ploidy, because the nuclei in binucleated cells were only separated to a very small extent by the preparation method used here.

Christie et al. (*7*) recognized a shift to higher ploidies in rat liver following DMN treatment. In their extensive study using Feulgen technique for DNA measurements and morphometry of the nuclei they also found a close relation between size and ploidy and advanced the hypothesis that the appearance of macronuclei may be the sign of a general shift to higher ploidies. The macronuclei phenomenon seen in mouse or rat liver in animals treated with various carcinogens like nitrofen (*8*), *o*-aminoazotoluene (*9*), pyrrolizidine alkaloids (*10*), aflatoxin (*11*), methylazoxymethanol acetate (*12*), *N*-nitrosomorpholine (*13*) or cadmium (*14*) may thus indicate that many of the hepatocytes are affected. Shifts to higher ploidy classes in liver of rodents exposed to carbon tetrachloride (*15*), *N*-nitrosomorpholine (*16*), and barbiturate (*17*) have been found by DNA measurements of individual cells. DMN, barbiturate, carbon tetrachloride and *N*-nitrosomorpholine gave increases in both nuclear and cellular ploidy. In a study exposing rats to lindane, Schulte-Hermann (*18*) found a reduction in binuclearity concomitantly with an increase in nuclear size, indicating an unchanged cellular ploidy.

The reversibility of the ploidy changes is another aspect which has not been clarified. The increase in ploidy following barbiturate treatment (*17*) is reversible, whereas DMN-induced changes can be seen months after a single treatment (own unpublished observations), and the macronuclei described in the literature are also often found after discontinuation of the treatment. Liver cell dysplasia, defined as cellular enlargement, nuclear pleomorphism and multinucleation, has been identified as a premalignant condition in humans (*19*).

Polyploidization is seen in other organ systems as an effect of carcinogens or suspected carcinogens; e.g., myleran induces polyploidy in lens epithelial cells (*20*) and hexachlorocyclohexane induces polyploidy in meristema root cells (*21*). High doses of griseofulvin and colchicine can induce polyploidy in several systems.

In vitro polyploidization is seen after treatment of a cell line with PUVA (*22*), a treatment now suspected to enhance skin carcinogenesis (*23*). Hamster cells exposed to asbestos fibers (*24*) and cell lines treated with different metals also cause such changes.

Many of these investigations are fragmentary and should be reexamined with improved methods like FCM. But there is obviously an association between carcinogens or cocarcinogens and polyploidization. It has not been shown whether the polyploidization effect of these substances is crucial to carcinogenesis, or whether it is merely an epiphenomenon. Cowell and Wigley (*25*) found evidence of polyploidization early in the carcinogenic process, and identified tetraploid cells in early stages of submandibulary gland carcinogenesis as probable precursors of malignant near-tetraploid tumor stemlines. When large numbers of malignant tumors are examined, the DNA content of stemlines is found to be mostly tetraploid or near-tetraploid and diploid or near-diploid (*26*).

This fact also indicates that polyploidization may take place, probably early in the process.

There are several possible mechanisms behind polyploidization. Colchicinlike substances may disrupt the spindle apparatus, and other agents may interfere by causing changes in the SH levels of the cells. This is probably the mechanism by which some metals like Hg and Cd disturb the mitotic process. The extensive redox changes made by many carcinogens may likewise be linked to such disturbances of SH level and may thereby affect the mitotic spindle, which is dependent on a certain level of SH groups for proper functioning. The PUVA-induced polyploidization is probably caused by DNA crosslinks (*27*). An increased functional load on the liver caused by hepatotropic agents is often accompanied by polyploidization. This may involve physiological mechanisms like adaptation to changed function or response to growth stimuli and may consequently be reversible.

FCM of Rat Hepatocytes during Early Stages of AAF Carcinogenesis

Throughout the observation period the ploidy of hepatocytes from animals on a control diet showed the normal distribution of diploid, tetraploid and octoploid DNA values. The lack of S-phase pulses and the low level of thymidine incorporation in short-term cultures of these hepatocytes is in accordance with the known low proliferative activity of the adult liver. The hepatocytes from rats fed AAF showed the same low proliferative activity measured by thymidine incorporation and judged from the microscopic appearance of the liver. In the FCM histograms, however, progressive changes were seen from week 12 of AAF feeding. These changes could be described as an increasing proportion of

pulses in the hypodiploid and hypotetraploid areas. At 16 weeks these nuclei with decreased fluorescence made up more than 50% of the nuclei (Fig. 3). Parallel samples stained with either ethidium bromide or mithramycin gave almost identical results. Provided there was a stochiometric binding of the fluorochromes to DNA under these conditions, these changes in the FCM histograms can be interpreted as the occurrence of aneuploid nuclei in increasing numbers in the liver during this state of the carcinogenic process. It is known that drugs can alter the binding of such dyes to DNA, and although this possibility cannot be completely excluded, most evidence supports the view that the changes in fluorescence represent a change in DNA content (*28*).

The progressive nature of these changes, starting at about the 10th week and comprising about 50% of the nuclei at the 16th week of exposure to AAF, fits well with the theory of a disturbed mitotic process. The average index of thymidine-labeled cells during this period was 0.15%, which implied that the cells proliferated at a rate of about 1% per day. Lesions due to nondisjunction or chromosome breaks during mitosis would thus accumulate at about the observed magnitude. Other investigators have put forward evidence for major alterations in the genotype of hepatocytes during the pretumor stage of hepatocarcinogenesis. Stich (*29*) found aneuploid DNA content of metaphases in the liver of rats exposed to dimethylaminoazobenzene. Barbason et al. (*30*) and Tates et al. (*31*) observed micronuclei in liver cells during an early phase of DMN-carcinogenesis.

This diversity of genotypes may be the cause of the well known diversity of phenotypes during early stages of hepatocarcinogenesis. Major DNA changes (*32*) may also play a role in hepatocarcinogenesis.

The kind of approach used here may be a useful model, and possibly a test system, for the detection of substances causing nondisjunction (turbagens) or chromosome breaks (clastogens). One can thus take advantage of the metabolic capacity of the rodent liver for activation of potential carcinogens and other harmful substances, and combine it with the use of FCM as a quick and relatively simple and reliable method.

FCM of Carcinogen-Induced Proliferative Lesions of the Liver

Thirty-one proliferative lesions in mice treated with DMN (see Methods) were classified as proliferative nodules, adenomas and hepatocellular carcinomas. The FCM histograms revealed characteristic patterns of ploidy and growth in the tumor stemlines. The nodules of hyperplasia showed a diploid and a tetraploid stemline, and the adenomas and most of the hepatocellular carcinomas were diploid (Fig. 4). Only two of the 14 hepatocellular carcinomas showed signs of aneuploid growth. The 15 rat liver tumors from AAF-barbiturate-exposed animals so far examined were all diploid.

Thus most of the liver tumors analyzed showed a low (2c) ploidy despite the enhanced polyploidization (in the mouse) and aneuploidy (in the rat) found in the pretumor stages. A clonal diploid growth of early liver tumors in mouse (*33*) and rat (*34*) has been found by microspectrofluorometry and chromosome counting, and has been interpreted as a sign of the fetal character of the liver tumors. Because most of the liver tumors in these experiments were isolated at an early stage of their progress, before any metastases were found, the material was probably not representative of the entire process. Some authors (*33*) have suggested that liver tumors progress from early differentiated diploid tumors to undifferentiated carcinomas containing aneuploid stemlines. The material presented here contained

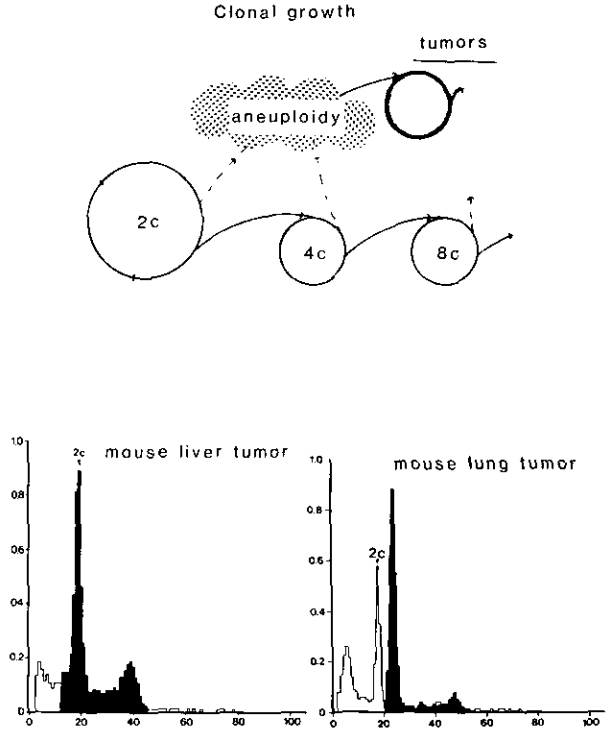

FIGURE 4. Clonal growth in a liver containing aneuploid and/or polyploid hepatocytes (top). The histograms of a hepatocellular carcinoma with a diploid stemline and a dominating S phase (left) (*4*) and a mouse lung tumor with an aneuploid stemline (right) (*26*) are shown.

only few undifferentiated carcinomas, but among these aneuploidy was found by the FCM measurements.

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