Flow cytometric analysis of neoplastic nodules and hepatocellular carcinomas induced by ciprofibrate in the rat

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Summary Alterations in DNA ploidy accompany hepatocellular carcinoma (HCC). However, changes in DNA content are also seen in regenerating liver and with increasing age. Thus, to investigate the role of DNA ploidy changes in development of HCC, flow cytometric DNA content determinations were done in a rat model system of peroxisome proliferator-induced HCC. Paraffin blocks of liver isolated from 18 Fisher 344 male rats fed ciprofibrate for 20 weeks (4), 40 weeks (4) or 20 months (10) were examined. Livers from age-matched control rats were also examined. From the 20 month ciprofibrate group, nine neoplastic nodules (NNs), 27 HCCs and four non-tumorous surrounding tissue controls (NTCs) were examined. Significant DNA tetraploid populations were seen in both the NNs and NTCs. A significant increase in the percentage of DNA diploid cells was observed in the NN samples. No significant difference in the percentage S-phase cells was seen. Emergence of cell populations with new DNA ploidy classes (8c or DNA aneuploid) as compared with NTCs was only seen in HCCs (7 of 27), and five of these seven were DNA aneuploid, as distinct from DNA tetraploid, populations. A total of 16 of 24 HCC samples that were adequate for cell cycle analysis had average percent S-phase greater than the mean of the NTCs plus three standard deviations. Although a direct role cannot be inferred, these results support the hypothesis that increases in the fraction of diploid cells is an important early event in the development of rat HCC and that further alterations in DNA ploidy and increased proliferative fraction accompany the development of HCC.

Keywords: rat hepatocellular carcinoma; DNA analysis; peroxisome proliferators; flow cytometry

Genotoxic and non-genotoxic carcinogens induce hepatocellular carcinomas (HCCs) in rats and mice (Reddy *et al.*, 1976; Pitot *et al.*, 1978; Williams, 1980; Maronpot and Boorman, 1982; Reddy and Lalwani, 1983; Goldsworthy *et al.*, 1986; Newberne *et al.*, 1987; Rao and Reddy, 1991*a*). In these experimental models, irrespective of the type of carcinogen used, the development of HCC is preceded by the formation of altered hepatocyte foci (AHF) and neoplastic nodules (NNs) (Farber and Cameron, 1980; Pitot and Sirica, 1980; Williams, 1980; Rabes, 1983; Scherer, 1984; Rao *et al.*, 1984, 1986; Rao and Reddy, 1991*b*). It is generally believed that HCCs arise from NNs, although some of them were shown to develop outside the nodules (Weisburger *et al.*, 1972). This also appears to be true of HCCs developing in cirrhotic livers in humans (Arakawa *et al.*, 1986; Terada *et al.*, 1989). The sequential morphological events that occur during the development of HCC fits ideally with the notion that a tumour acquires the malignant phenotype through sequential multiple genetic changes (Friend *et al.*, 1988). These changes include abnormal ploidy, oncogene activation and suppressor-gene inactivation (Cho and Vogelstein, 1992). The molecular events that take place during the progression of liver lesions from normal cells to HCC are poorly characterised. In the majority of studies employing genotoxic agents, an increase in the percent DNA diploid or $2n$ fraction of cells has been reported (Schwarze *et al.*, 1984; Styles *et al.*, 1985; Sarafoff *et al.*, 1986; Denielsen *et al.*, 1988; Haesen *et al.*, 1988), as has the appearance of DNA aneuploid populations in HCC. However, for treatment with non-genotoxic agents such as ciprofibrate, the nature and temporal appearance relative to AHFs, NNs and HCCs of DNA ploidy alterations has been less well studied. In the present study, we have examined NNs, HCCs and adjacent non-tumorous liver from rats treated with ciprofibrate, a potent hypolipidaemic peroxisome proliferator, for changes in cellular DNA content using flow cytometry methodology.

Materials and methods

Induction of liver tumours

Eighteen male F-344 rats (Charles River Breeding Laboratories, Wilmington, MA, USA) at approximately 8 weeks of age (80–100 g) were started on a diet (Purina rat chow) containing 0.025% (w/w) ciprofibrate (Sterling-Winthrop Research Institute, Rensselaer, NY, USA). Animals were sacrificed after 20 weeks ($n = 4$), 40 weeks ($n = 4$) or 20 months ($n = 10$) on the ciprofibrate diet. As control, six animals were fed control diet (rat chow with no ciprofibrate) for the same periods of time ($n = 2$ at each time point). At necroscopy, multiple representative sections from each liver were fixed in neutral buffered formalin and processed for light microscopy.

Flow cytometric measurements

A haematoxylin–eosin (H&E)-stained section from each paraffin block was examined before cutting 3–5 40-μm sections for isolation of nuclei. For control animals and ciprofibrate-fed animals at 20 and 40 weeks, there were no lesions or foci large enough for isolation and the entire tissue was analysed (multiple samples were analysed per animal). For ciprofibrate-fed animals at 20 months, the H&E section was examined to identify areas that were predominantly NN, HCC or normal tissue. Careful scoring of the block was then done to allow isolation and separate processing of NNs, HCCs or surrounding normal areas when sections were cut. Samples were then processed for flow cytometric DNA analysis by the method of Hedley *et al.*, 1983), with slight modifications. Briefly, sections were deparaffinised in xylene and rehydrated through an alcohol series. The material was then incubated in distilled water overnight, minced, digested in 2 ml of 0.5% pepsin (Sigma, St Louis, MO, USA) in 0.9% sodium chloride (pH 1.5) at 37°C for 30 min, sieved through a 37 μm mesh, and the pH neutralised. The sample was washed in phosphate-buffered saline (PBS) and the concentration adjusted to 10^6 cells ml^{-1}. The cell suspension was then treated as follows: washed, incubated for 3 min on ice in PBS containing 0.1% Triton X-100 (Sigma), washed, incubated at 37°C for 20 min in PBS containing 180 units

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RNAase ml^{-1} (Sigma), washed and resuspended in PBS containing 0.3% polyethylene glycol (Research Products International, Mount Prospect, IL, USA) and 50 μg ml^{-1} propidium iodide (Calbiochem, San Diego, CA, USA). All samples were examined microscopically for the presence of doublets, and if more than 5% were seen samples were syringed through a 21 gauge needle until the proportion of doublets was less than 5%. Additionally, doublets were electronically removed from analysis by gating on peak *vs* integral red fluorescence signals. All cell samples were analysed on the Coulter Electronics Profile II flow cytometer with PowerPak option (Coulter Electronics, Hialeah, FL, USA) at the Veterans Administration Lakeside Medical Center, collecting more than 50 000 events per sample. All samples were filtered through a 37 μm nylon mesh just before analysis. Laser excitation was 15 mW at 488 nm and the standard optical filter configuration was used for red fluorescence light detection (488 nm dichroic, 457-502 nm long-pass laser blocking, 550 nm dichroic, 600 nm dichroic and 635 nm bandpass filters). Cell cycle analysis was done using the Modfit analysis routines (Verity Software House, Topsham, ME, USA), employing a single Gaussian function for each of the 2n, 4n, 8n etc. peaks, single-cut debri and rectangular S-phase functions. No population was classified as DNA aneuploid unless two distinct G_0/G_1 peaks were evident in the DNA content distribution (Hiddemann *et al.*, 1984). A DNA tetraploid population was defined as having a DNA index (DI) of 1.8–2.2 and a DNA octoploid population as having a DI of 3.6–4.4. A DNA tetraploid population was defined when the percentage of G_2/M, or tetraploid G_0/G_1 peak, was greater than 15% of the sum of [%G_0/G_1 + %S + %G_2/M (or DNA tetraploid peak)] (Rabinovitch, 1993). A DNA octoploid population was defined in an analogous manner. Since in these conventional DNA ploidy definitions, DNA ploidy class is defined by an artificial cut, abrupt changes in the percentage of a given DNA ploidy class can occur. For example, a sample with 16% 4n cells will be reported with both DNA diploid and tetraploid populations, whereas a sample with 14% 4n cells will be reported as 100% DNA diploid. Thus, the percentage of 2n, 4n and 8n cells are also reported. Since the DNA diploid or tetraploid G_2/M population overlaps with the DNA tetraploid or octoploid G_0/G_1 respectively, proliferative fraction (%S + %G_2/M) data for each DNA ploidy population are not reported. However, no overlap of the S-phase fraction of each ploidy class occurs and, thus, the weight-averaged S-phase was calculated for each sample. Statistical comparisons of the percentage of DNA diploid and percentage of 2n fractions were done using the two-tailed Student's *t*-test.

Results

Livers from all control animals and the 20 week ciprofibrate-fed animals appeared normal and did not contain any AHF, NN or HCC lesions. Examination of the livers of the 40 week ciprofibrate-fed animals revealed only AHF, but no nodules or HCC. These lesions were too small for separate isolation for flow cytometric analysis. The livers of all ten rats fed ciprofibrate for 20 months contained multiple grossly visible lesions. Histological examination of liver sections showed AHF, NN and HCC (Figures 1 and 2). The morphological features of these different lesions have been described previously (Rao *et al.*, 1990). Representative NNs, HCCs and NTC liver from each animal, based on H&E sections, were carefully selected for flow cytometry. A total of four NTCs, nine NNs and 27 HCCs were isolated.

The results for all animals fed control diet, regardless of age, and for the 20 week and 40 week ciprofibrate-fed animals was the same, showing either a predominant or exclusively DNA diploid population. The range in the per cent DNA diploid fraction for these samples was from 65–100% (data not shown).

Figure 3a and b shows typical results for an NTC and an NN sample, respectively, from an animal-fed ciprofibrate for

20 months. Prominent DNA tetraploid and DNA diploid populations are seen. All nine NNs had similar DNA ploidy patterns and the results are summarised in Table I. In no case were cell populations with a DI greater than 2.2 (>4c DNA content) seen, nor were any DNA aneuploid (as distinct from DNA heteroploid) populations observed. A statistically significant ($P = 0.007$) increase in the DNA diploid fraction was seen in NNs (mean = 50% ± 4%) as compared with non-tumorous adjacent areas (mean = 33% ± 6%). This difference is also reflected in the changes in average percentage of 2n, 4n and 8n cells with a statistically significant ($P = 0.03$) difference in percentage of 2n of NNs as compared with the NTCs.

Figure 4 shows three representative results for HCC specimens. A summary of HCC analyses are presented in Table I. In Figure 4a a sample with multiple heteroploid cell popula-
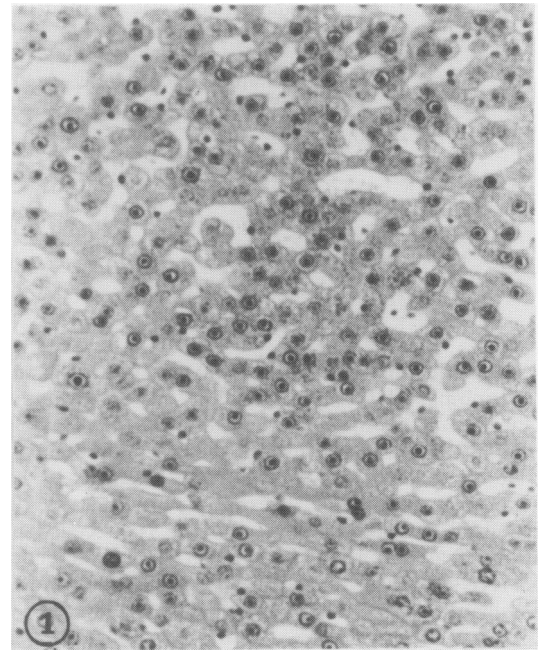

Figure 1 Morphology of representative neoplastic nodules used for flow cytometric analysis. (H&E × 150).
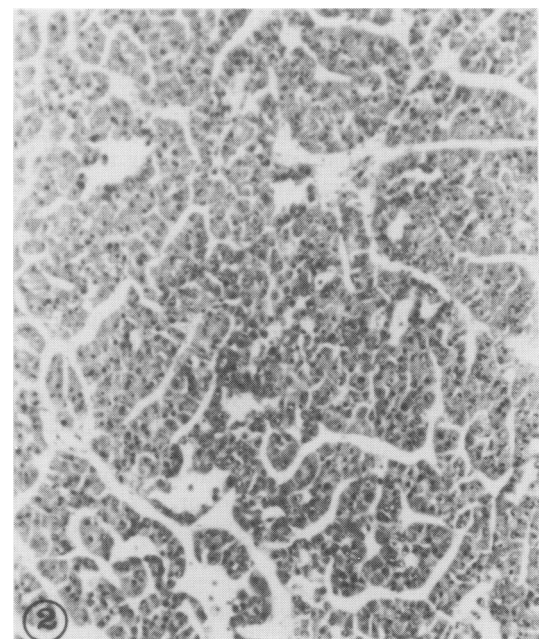

Figure 2 Morphology of representative hepatocellular carcinoma used for flow cytometric analysis (H&E × 110).

tions having DI of 1.0 (2c), 2.0 (4c), and 4.0 (8c) is shown. In no samples were significant populations having DI greater than 4.4 (8c) observed (see insert in Figure 4a). In Figure 4b, an HCC sample with four DNA ploidy populations (DI = 1.0, 1.8, 2.0 and 2.2) is shown. It should be noted that the results for all NN and NTC samples were consistent with DNA diploid or tetraploid populations. Seven of 27 HCC samples exhibited cell populations with DNA ploidy values (DI) distinct from those seen in NNs and NTCs. Additionally, five of seven of these HCC samples exhibited DNA aneuploid populations as distinct from DNA heteroploid (i.e consistent with 2c, 4c, 8c etc. DNA content). This is in contrast to the NN samples and NTC adjacent areas in which all populations were consistent with DNA heteroploid populations. Again, a statistically significant increase in the DNA diploid fraction ($P = 0.003$) and in the percentage of $2n$ ($P = 0.004$) as compared with surrounding NTC tissue was seen.

Evaluation of the weight-averaged per cent S-phase cells is shown in Table I. A trend to increasing average per cent

S-phase was seen in the progression from NTCs to NNs to HCCs with statistically significant differences seen in the HCCs vs NTCs ($P = 0.003$) and HCCs vs NNs ($P = 0.01$) groups. Within the NN samples, three of nine had average per cent S-values greater than the NTC mean plus three standard deviations as compared with 16/24 in the HCC group.

Discussion

In this study, DNA ploidy alterations were investigated in a rat model system of peroxisome proliferator-induced hepatocellular carcinoma. Examination of nine 'premalignant' NNs did not reveal the appearance of any new DNA ploidy classes as compared with surrounding non-tumorous controls (NTCs). Although, a significant increase in the fraction of DNA diploid or $2n$ DNA content cells was seen as compared with NTC. In examination of 27 hepatocellular carcinoma samples (HCC), seven exhibited the appearance of
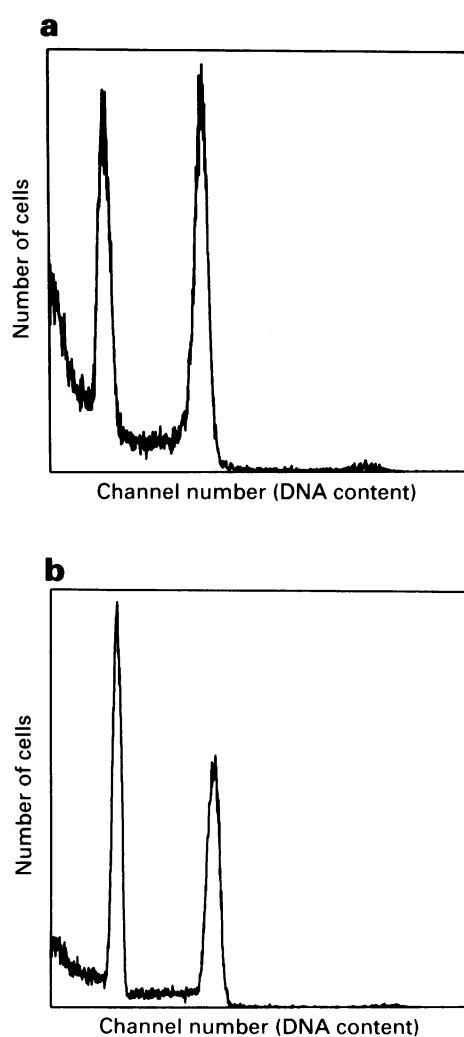

Figure 3 Flow cytometric distributions showing number of events vs red fluorescence (DNA content) for representative samples of non-tumorous surrounding tissue (a) and neoplastic nodule (b).
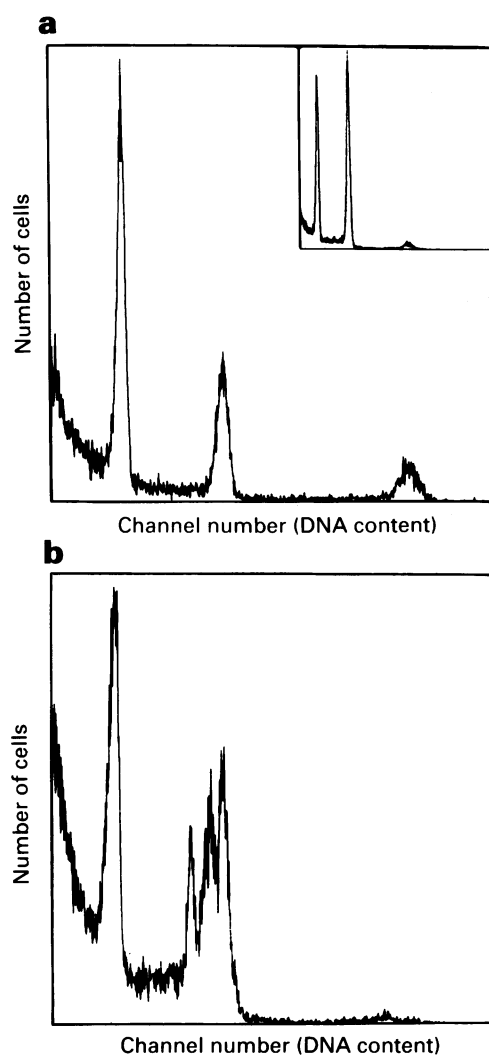
Figure 4 Flow cytometric distributions showing number of events vs red fluorescence (DNA content) for representative HCC samples. Insert in (a) is HCC sample analysed at a lower amplifier gain setting to illustrate the lack of significant cells with DNA content greater than 8c.

Table I Summary of DNA ploidy and cell cycle data for NTC, NN and HCC samples

Sample	Average per cent S	Per cent diploid[a]	Per cent 2n	Per cent tetraploid[a]	Per cent 4n	Per cent octaploid[a]	Per cent 8n
NTC ($n = 4$)	2.5 ± 0.9	33 ± 6	33 ± 7	68 ± 6	65 ± 6	–	2 ± 1
NN ($n = 9$)	4.4 ± 3.7	50 ± 4	50 ± 4	50 ± 4	49 ± 5	–	1 ± 1
HCC ($n = 27$)	9.9 ± 9.1[b]	52 ± 13	52 ± 14	46 ± 15	42 ± 17	3 ± 6	4 ± 5

[a] As defined in Materials and methods. [b] Only 24/27 HCC samples were adequate for S-phase determinations.

new DNA ploidy classes as compared with surrounding non-tumorous areas. The same increase in DNA diploid or 2n DNA content fraction as was seen in the NNs was observed in HCCs. A trend to increasing S-phase cells with progression from NTC to NN to HCC was seen with three of nine NN and 16/24 HCC samples having average per cent S greater than the NTC mean plus three standard deviations. Thus, these results do indicate an association of altered DNA ploidy and increased per cent S-phase with progression to HCC following treatment with a non-genotoxic peroxisome proliferator.

The predominance of a DNA diploid population in all control samples (20 weeks, 40 weeks or 20 months on control diet) and in the 20 and 40 week ciprofibrate-fed animals is in contrast with previous reports (Romagna and Zbinden, 1981; Schwarze et al., 1984; Styles et al., 1985; Styles et al., 1988; Wang et al., 1990) in which DNA tetraploid populations were predominant. In these previous reports, either image technology or cell isolation procedures that enriched the parenchymal cells were used. However, in this study, no enrichment of parenchymal cells was done. Thus, in these samples in which the entire tissue was analysed (no isolatable lesions were present), the results probably reflect the presence of non-parenchymal (diploid) cells within the liver. Interestingly, examination of the non-tumorous areas surrounding the NN and HCC lesions in the 20 month ciprofibrate-fed animals exhibited a more expected pattern with an average per cent DNA diploid fraction of 33%. When comparing the numbers from this study with others, it must be remembered that these results reflect nuclear ploidy and not cellular ploidy. Since this value is still somewhat on the high end of reported values for normal rat liver, inclusion of some non-parenchymal cells cannot be ruled out. Nonetheless, this is still a dramatic difference as compared with the normal control animals. Whether this reflects an effect of long-term ciprofibrate feeding of the animals or an effect of the NN or HCC on the surrounding normal tissue remains to be seen. Although less likely differences in the recovery of parenchymal vs non-parenchymal cells in controls vs ciprofibrate-fed animals cannot be ruled out. The preferential selection of population has been reported when using the Hedley method (Koss et al., 1989).

In the isolation of NN and HCC, careful dissection was done to ensure that no normal tissue was included. Thus, the problems discussed above concerning parenchymal vs non-parenchymal cells does not apply. In both NNs and HCCs, a significant increase in the per cent DNA diploid fraction was seen as compared with NTC. It should be noted that even if inclusion of non-parenchymal cells in the NTC did occur the effect would be to mask rather than increase the differences seen in the NNs and HCCs. As such, this shift appears to be real and not an artifact of sample isolation and preparation. This result is similar to that seen in AHF or NN following treatment with a number of genotoxic agents (Schwarze et al., 1984; Styles et al., 1985; Sarafoff et al., 1986; Denielsen et al., 1988; Haesen et al., 1988). Additionally, treatment with 2-acetylaminofluorene alone (Klose et al., 1989) or N-nitrosomorpholine (Romagna and Zbinden, 1981) results in an increased diploid fraction. In contrast, studies with dimethylnitrosamine treatment have reported a shift to an increased tetraploid population (Digernes, 1983; Carlson and Abraham, 1985). Thus, although there may be a few exceptions, these results, as with the majority of studies employing genotoxic agents, would be consistent with a significant increase in the diploid fraction of cells being an early event in the development of HCC in rats following treatment with ciprofibrate. The average per cent S of the NNs, although higher, was not statistically different than the surrounding NTC.

The DNA ploidy alterations seen in the HCCs are similar to other reports (Stich, 1960; Nowell et al., 1967; Becker et al., 1971, 1973; Mori et al., 1982; Digernes, 1983; Styles et al., 1985; Denielsen et al., 1988; Haesen et al., 1988). However, the frequency of DNA aneuploidy in this model of HCC development is significantly lower (7/27) than has been reported in a number of studies using genotoxic agents (Stich, 1960; Nowell et al., 1967; Becker et al., 1971, 1973; Mori et al., 1982). Although loss of DNA aneuploid populations has been seen with analysis of paraffin samples (Koss et al., 1989), Digernes (1983) has also reported a low frequency of aneuploidy in HCC, and a high degree of variability, even among similar lesions in the same animal, has been reported (Denielsen et al., 1988). The observation that progression to HCC was frequently accompanied by an increased per cent S-phase is not unexpected as increased proliferative fraction is a negative prognostic indicator in a number of malignancies (Bauer et al., 1993). However, it is interesting to note that the increased frequency of lesions with higher per cent S and altered DNA ploidy occurred together in this model system. Correlation or association of increased per cent S in DNA aneuploid tumours or karyotypically unstable populations has been observed (Chen et al., 1991; Waldman et al., 1991; Steiner et al., 1993), and it has been hypothesised that genetic changes may provide a proliferative advantage (Volpe, 1990) and that accumulation of alterations are associated with progression (Friend et al., 1988). However, it should be noted that the low frequency of DNA aneuploid HCC in this model system does not imply that karyotypic or genetic instability is not still driving the process of progression to HCC (Sudilovsky et al., 1991).

Only limited conclusions can be drawn about the underlying mechanisms of the genetic or DNA changes seen, since the analyses done in this study were at the gross DNA ploidy level, being insensitive to chromosomal changes involving fewer than several chromosomes. However, certainly in liver, a differentiated feature is the generation of tetraploid cells implying that with differentiation in liver there is a block in normal cytokinesis following cell replication, leading to the generation of DNA tetraploid and higher ploidy populations. With progression to malignancy there is a degree of loss of this differentiated feature or block and an increase in the diploid fraction of cells. This data would be consistent with this block in normal cytokinesis no longer functioning in the malignant cells. The appearance of non-integer DNA aneuploid populations does not occur until later in neoplastic progression when presumably, as with other tumour types, the loss of karyotype stability generates a wide range of both chromosomal structural and numerical changes. Elucidation of the primary mechanism (non-disjunction, etc.) underlying this instability awaits chromosomal studies.

DNA aneuploidy is common in human HCC and appears to be of prognostic value in patients (Popp and Marsman, 1991). The data examining DNA ploidy alterations in early lesions during the development of human HCC is limited, with reports of DNA aneuploidy being frequent in human AHF (Thomas et al., 1992) and others reporting primarily diploid cells in AHF (Hoso and Nakanuma, 1991). However, the data suggest an earlier appearance of DNA aneuploidy in human HCC than in HCC in rats induced either by PP or genotoxic agents. It is mere speculation to hypothesise why this might be the case. Access to early lesions in the rat model system is far easier, and thus, even 'early' lesions in human may be further progressed than the early lesions that were examined in the rat. Clearly, the pattern of alterations in rat HCC varies with causative agent and differences in the primary causative agent might be related to the varying course of DNA alterations seen in human cells as well. Additionally, human cells are significantly more resistant to malignant transformation in vitro than rodent cells, particularly with regard to immortalisation (Shay et al., 1989), and thus, greater genetic alteration, manifested as DNA aneuploidy, may occur sooner. However, it must be stated again that generation of DNA euploid malignant clones or foci is not inconsistent with underlying genetic instability (Sudilovsky et al., 1991).

In conclusion, induction of HCC by the non-genotoxic peroxisome proliferator, ciprofibrate, in rat results in a significantly increased fraction of diploid cells in both NN and HCC as compared with surrounding non-tumorous tissue. As discussed above, this result is similar to results with a

number of other hepatocarcinogens in the rat, and points to this being an important early event in the development of HCC in the rat. These early lesions did not exhibit a significantly increased per cent S-phase cells. However, careful assessment of the role of these changes in the earliest AHF or NN and comparison with the parenchymal cells of age-matched control animals or surrounding normal tissue will require alternative techniques such as image analysis. As has been generally found, HCC lesions exhibited an increased frequency of DNA ploidy alterations, a result consistent with continued genetic instability with progression to HCC. Additionally, a correlation of increased per cent S in the HCC lesions was also found.

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