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Cytophotometric DNA analysis of hepatocellular carcinoma with Mallory bodies

Masahiro Hoso and Yasuni Nakanuma
Second Department of Pathology, Kanazawa University School of Medicine, Kanazawa 920, Japan

Summary. In order to clarify the pathological significance of Mallory body (MB) formation in human hepatocellular carcinoma (HCC), cell nuclear deoxyribonucleic acid (DNA) content was measured microspectrophotometrically in 20 autopsied cases of HCC associated with cirrhosis and bearing many MBs. According to the degree of dispersion, the DNA histogram was classified into type I (diploid pattern), type II (hyperploid pattern) and type III (aneuploid pattern). Non-neoplastic hepatocytes of normal livers and of cirrhotic areas of the 20 HCC cases showed generally a diploid pattern (type I). In contrast, MB-positive HCC cells showed more hyperploidy or aneuploidy (type I: 0%; type II: 35%; and type III: 65%) compared with MB-negative HCC cells (type I: 25%; type II: 50%; and type III: 25%). These data suggest that MB formation in HCC is accompanied by a constant change of DNA content of HCC cells, though the causal relation between them is only speculative. Two separate HCC nodules in the same liver, both of which contained many MB-positive cells, showed the same type of DNA histogram pattern, suggesting the possibility that they were of a monoclonal origin and had spread discontinuously in the liver.

Key words: Hepatocellular carcinoma – Mallory body – DNA-histogram

Introduction

Mallory bodies (MBs) were once been regarded as a characteristic finding of alcoholic hepatitis (Borenfreund et al. 1979; French 1981, 1983). They are, however, not strictly specific for alcoholic liver cell injury, and a variety of diseases such as hepatocellular carcinoma (HCC), primary biliary cirrhosis and Wilson disease have been found to show MBs (Borenfreund et al. 1979; French 1981, 1983). MBs have been produced experimentally in mice by prolonged griseofulvin administration (Denk et al. 1979). Although the chemical characteristics and structures of MBs have been clarified, their biological significance and pathogenesis in human hepatobiliary diseases are still controversial. Our previous study mapping of MB-positive cells within human HCC suggested that MB-positive HCC cells showed clonal proliferation (Nakanuma and Ohta 1986a). We have also seen 4 cases of small HCC composed exclusively of MB-positive cells, supporting this suggestion (Nakanuma and Ohta 1984; Nakanuma et al. 1986b). Although these data suggest that MB formation is possibly a heritable cellular change of HCC, more data are necessary to confirm this suggestion.

Bohn and Sandritter (1975) reported that determination of cell nuclear deoxyribonucleic acid (DNA) content was meaningful in the evalution of the biological activities, degree of differentiation and proliferating capacity of tumour cells. The present study was aimed to further elucidate the biological and pathological significance of the MB phenomenon in HCC by measuring nuclear DNA content.

Materials and methods

Twenty human autopsy livers with HCC, bearing many MBs, were selected from 193 cases autopsied in our Department between 1972 and 1987. All of these cases were associated with liver cirrhosis (Table 1). There were 15 males and 5 females and the age ranged from 43 to 85 yr (mean: 64.1 yr). Two to 10 specimens were obtained from neoplastic as well as non-neoplastic liver in each case, and were fixed in 10% formalin and then embedded in paraffin. As to the distribution of MBs in HCC (Nakanuma and Ohta 1986a), 8 cases showed a diffuse pattern (almost all HCC cells in the liver contained MBs) and

Offprint requests to: Y. Nakanuma

Table 1. Presentations of 20 cases of HCC bearing MBs

Case no.	Age	Sex	HCC (Macro)	Grade of HCC (Edmondson)	LC	Etiology of LC	Distribution of MBs in HCC
1	46	F	Nodular	III	+	B	Diffuse
2	68	M	Nodular	II	+	NANB	Diffuse
3	45	M	Nodular	II	+	NANB	Diffuse
4	57	M	Massive	II	+	NANB	Diffuse
5	53	M	Nodular	II	+	Alco	Diffuse
6	58	M	Nodular	III	+	Alco	Diffuse
7	68	F	Small[a]	II	+	NANB	Cluster
8	60	M	Nodular	III	+	Alco	Cluster
9	85	M	Nodular	II	+	NANB	Cluster
10	75	M	Nodular	II	+	Alco	Cluster
11	72	M	Nodular	II	+	Alco	Cluster
12	57	M	Small[a]	II	+	Alco	Cluster
13	68	M	Nodular	III	+	B	Cluster
14	70	F	Nodular	II	+	B + Alco	Cluster
15	60	F	Nodular	II	+	B	Cluster
16	74	M	Nodular	III	+	Alco	Cluster
17	59	M	Nodular	II	+	NANB	Cluster
18	43	M	Nodular	II	+	Alco	Cluster
19	60	F	Nodular	II	+	NANB	Cluster
20	60	M	Nodular	II	+	NANB	Cluster

HCC = hepatocellular carcinoma, MBs = Mallory bodies, No. = number, M = male, F = female, LC = liver cirrhosis, NANB = non A non B virus, B = hepatitis B virus, Alco = alcohol

[a] Diameter of tumor size < 2 cm

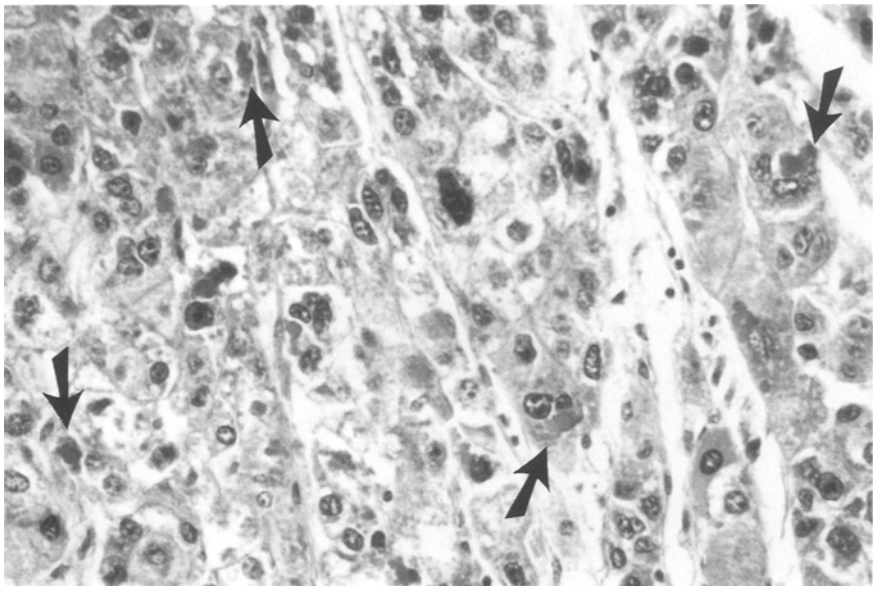

Fig. 1. Many carcinoma cells contain Mallory bodies (*arrows*) in their cytoplasm. Hepatocellular carcinoma. HE, ×325

the remaining 12 had a clustering pattern (almost all HCC cells in some parts of HCC contained many MBs) (Fig. 1). HCC was histologically graded according to Edmondson and Steiner (1954). As a control, 9 normal human autopsy livers from our recent file, showing the similar age and sex distribution of the 20 HCC cases mentioned above, were used.

Alternative serial sections, 5 μm and 12 μm thick, were cut from each paraffin block. The 5 μm section was stained with haematoxylin and eosin, and the adjacent 12 μm thick paraffin section in which the possibility of cutting or overlapping of nuclei of the hepatocytes and HCC cells measured was reduced, was prepared for Feulgen staining (Koike et al. 1982, 1985). The sections were deparaffinized by xylene and ethanol, and hydrolyzed in three steps, 1 N-HCl at 37° C for 1 min, 1 N-HCl at 60° C for 7 min and 1 N-HCl at 37° C for 1 min. These sections were then rinsed with distilled water and immersed in the staining solution consisting of 0.5 ml of Schiff's solution, 8.5 ml of 0.1 M Sorensen glycin buffer (pH 2.28) and 1 ml of 15% sodium metabisulfite solution (9:1) for 10 min, subsequently rinsed with running water for 30 min, dehydrated in

ethanol and xylene and finally mounted. These stained sections were then postirradiated for one week by green excitation light of mercury-vapor lamp (Toshiba HB-1048HB, Toshiba, Tokyo, Japan) or for 3 days under the sunlight to eradicate non-specific fluorescence (Kagawa 1981; Koike et al. 1982, 1985). The microspectrophotometric measurement was carried out under a fluorescent microscopy (Standard type, Zeiss, FRG) with a microspectrophotometer (Nikon P1, Nippon Kogaku Corp, Tokyo, Japan). It was found that identification of MB was impossible in Feulgen stained sections under either light or fluorescent microscope. Thus carcinoma cells in the areas of HCC containing numerous MBs which were identified by the adjacent HE-stained section, were measured by spectrophotometry. MB-negative HCC areas were measured similarly in 12 HCC cases, though there were no MBs-free areas in the HCC tissue examined in the remaining 8 cases showing a diffuse distribution pattern of MBs. In each specimen, the DNA content was measured in about 200 MB-positive and/or MB-negative HCC cells, and also in about 200 non-neoplastic hepatocytes from normal livers as well as cirrhotic areas of HCC cases. Nuclear DNA content was also measured in more than 50 lymphocytes in each section of HCC cases and normal livers, and the modal value of lymphocytic content thereby determined, it was defined as diploid (2c) (Bohn and Sandritter 1975; Koike et al. 1982, 1985). The DNA content was displayed as a histogram by a computer (PC 9801, NEC, Tokyo, Japan) connected to the spectrophotometer.

The DNA distribution pattern was grouped into three types according to Sugimachi et al. (1984) and Inokuchi et al. (1983) with a slight modification. Type I (diploid pattern): distribution with a prominent peak in the 2c region and with a narrow range of dispersion limited to the 4c region; type II (hyperploid pattern): distribution with a relatively high peak at the polyploid region beyond the 2c region; type III (aneuploid pattern): a rather low peak beyond the 2c region or multiple peaks, with a broad range of dispersion and a number of aneuploid cells. A representative DNA histogram of these three types is respectively shown in Fig. 2.

Statistical analysis was performed using the chi-square test and Fisher's exact test, and a value of $p<0.05$ was considered to be statistically significant.

Results

DNA histograms of normal livers and of noncancerous cirrhotic portions of the HCC cases was of type I. In contrast, the DNA histogram patterns of HCC cells (MB-positive and MB-negative cells) were variable from type I to type III as shown in Table 2. Generally, MB-positive HCC cells showed more hyperploidy and aneuploidy when compared with MB-negative HCC cells. That is, in MB-positive HCC cells, type II was found in 7 cases (35%), type III in the remaining 13 cases (65%) though there were no cases showing type I. In MB-negative HCC areas, however, type I was in 3 cases (25%), type II in 6 cases (50%), and type III in the remaining 3 cases (25%). The proportion of types II and III in MB-positive HCC cells was higher than that in MB-negative HCC cells ($p<0.05$). The proportion of type III in MB-positive HCC cells was also higher than that in MB-nega-

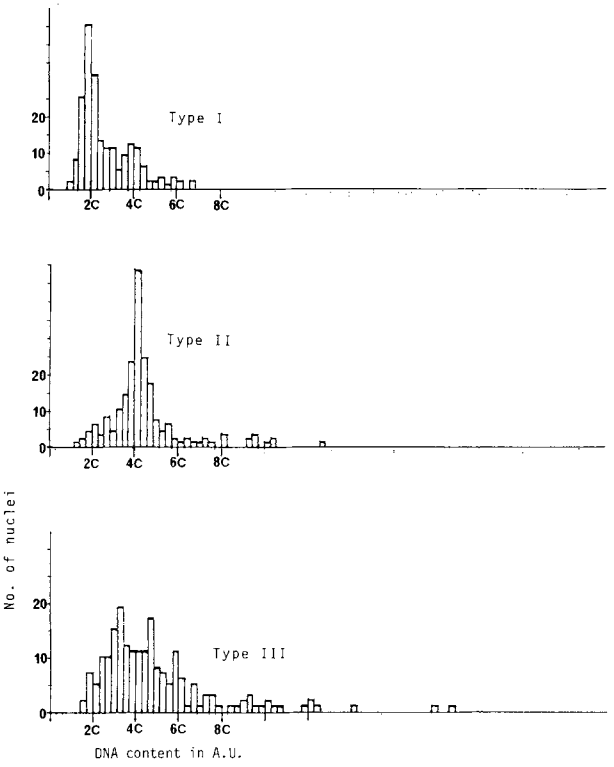

Fig. 2. Types of nuclear DNA histogram. Type I, diploid pattern (case 10, hepatocellular carcinoma areas without Mallory bodies). Type II, hyperploid pattern (case 21, hepatocellular carcinoma areas without Mallory bodies). Type III, aneuploid pattern (case 4, hepatocellular carcinoma areas with Mallory bodies)

Table 2. Types of DNA histogram pattern in 20 cases of HCC

Case no.	MB-positive HCC area	MB-negative HCC area
1	III	–
2	III	–
3	II	II
4	III	III
5	II	I
6	II	–
7	II	II
8	III	I
9	III	–
10	III	II
11	II	II
12	III	–
13	III	–
14	III	–
15	III	III
16	III	III
17	II	–
18	III	II
19	II	I
20	III	II

MB = Mallory body, HCC = hepatocellular carcinoma, – = MBs negative areas are not found in HCC tissue examined

Table 3. Correlation of types of DNA histogram pattern in MB-positive and MB-negative HCC areas in the same case

		MB (−) HCC area		
		Type I	Type II	Type III
MB (+) HCC area	Type I	0	0	0
	Type II	2	3	0
	Type III	2	2	3

MB = Mallory body, HCC = hepatocellular carcinoma, no. = number of cases

tive HCC cells ($p < 0.05$). It was, however, extremely difficult to deduce the DNA histogram pattern from microscopic findings in MBs-positive HCC areas or MBs-negative HCC areas. There was no clear correlation between DNA typing and other histological features, including grading of HCC in MB-positive HCC cells.

Comparison of types of DNA histogram pattern between MB-positive and MB-negative HCC areas in the same liver was done in 12 cases (Table 3). Six cases showed the same type of DNA histogram pattern in MB-positive and MB-negative areas, while in the remaining 6 cases, MB-positive HCC areas showed higher graded types, that is, more hyperploid and aneuploid patterns, than MB-negative HCC areas did.

DNA histogram patterns of two separate carcinomatous portions in the same liver, both of which contained many MB-positive HCC cells, were examined in 7 cases. Six of them showed the same DNA histogram pattern. That is, both of two different parts of MB-positive HCC of case 3 showed type II, those of case 4 showed type III, those of case 6 showed type II, those of case 14 showed type III, and those of case 18 did type III. However, DNA histogram pattern was different in case 7, that is, one part showed type II and another part type III.

Discussion

The nuclear DNA content in HCC cells has been examined using microspectrophotometry on imprint or smear preparations from fresh, unfixed tumours, as well as on thick paraffin sections from formalin fixed HCC tissue (Ezaki et al. 1988; Kuo et al. 1987; Kagawa 1981). It is well known that HCC cells show a variable distribution patterns of nuclear DNA content (Ezaki et al. 1988; Kuo et al. 1987; Kagawa 1981).

In this study, the nuclear DNA content of non-neoplastic hepatocytes and HCC cells with and without MBs was measured and their nuclear DNA-histograms were compared with each other. It was found that while non-neoplastic hepatocytes showed a diploid pattern, HCC cells tended to show a hyperploid and aneuploid pattern, and also that MB-positive HCC cells were more hyperploid and/or aneuploid when compared with MB-negative HCC cells. The former finding has been already reported in the English and Japanese literatures (Ezaki et al. 1988; Kuo et al. 1987; Kagawa 1981), but the latter finding is quite new to our knowledge. Bohn and Sandritter (1975) reported that the vast majority of the malignant tumours exhibited a more or less increased nuclear DNA content and variable ploidy patterns, and also that increased nuclear DNA content and aneuploid patterns in DNA histogram were rather dependable signs of malignancy of tumour cells. In fact, the DNA pattern of malignant cells usually reflects the clinical outcome in gastric and oesophageal carcinomas (Inokuchi et al. 1983; Sugimachi et al. 1984). In this sense, MB-positive HCCs might be more malignant than MB-negative ones. Prospective survey of the patients with MB-positive as well as MB-negative HCCs using surgically resected specimens seems necessary to resolve this issue.

French (1981), Tazawa et al. (1983) and Borenfreund et al. (1979) using experimental animals, demonstrated that MB is a structural phenotypic cellular change in experimental hepatocarcinogenesis. It was found, in the present study, that separate HCC nodules containing many MB-positive carcinoma cells in the same liver tended to show the same type of DNA histogram pattern, suggesting that the MB-positive HCC cells in these separate nodules were possibly of an unicentric origin with clonal proliferation and subsequent intrahepatic discontinuous spread. In this sense, MB formation may be a heritable phenotypic marker in human HCC as suggested in experimental carcinogenesis (Borenfreund et al. 1979; French 1981; Tazawa et al. 1983). It was also found in the present study that the DNA pattern was different between MB-positive and MB-negative areas in the same liver, suggesting that they were of a different clonal origin.

It is also possible that the increase of nuclear DNA content is an epiphenomenon associated with MB formation. It is well known that MB formation reflects an alteration of the cytokeratin filament and is thereby associated with derangement of cytoskeletal architecture (Borenfreund et al. 1979; French 1981, 1983; Tazawa et al. 1983). This cytoskeletal derangement with MB formation may be associated with disturbance of cell kinetics of HCC cells followed by increased DNA content

showing a hyper- and/or aneuploidy pattern in the absence of cell divisions. However, the primary initiating mechanism leading to MB formation in HCC cells is unknown.

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