

Mallory Bodies in Hepatocytes of Alcoholic Liver Disease and Primary Biliary Cirrhosis Contain N^{ϵ} -(Carboxymethyl)lysine-Modified Cytokeratin, but not those in Hepatic Carcinoma Cells

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Mallory bodies (MBs) are intracytoplasmic bodies seen in hepatocytes of alcoholic liver disease, primary biliary cirrhosis and hepatocellular carcinoma. However, the mechanism of MB formation has not been fully understood. Proteins could be modified to advanced glycation end products (AGEs) after long-term incubation with reducing sugar. AGEs are known to accumulate in several tissues in aging and age-enhanced disorders. To study the possible glycation process in the formation of MBs, hepatocytes of 80 human liver tissues with MBs were subjected to immunohistochemical analyses with five AGEs, two markers for oxidative stress proteins (OSPs) and four stress-response proteins (SRPs). MBs in hepatocytes of primary biliary cirrhosis and alcoholic liver disease were strongly positive for N^{ϵ} -(carboxymethyl)lysine (CML) and weakly positive for pyrrolidine. MBs in hepatocellular carcinomas were negative for both CML and pyrrolidine. No significant immunoreactivity was detected in MBs for other AGEs, such as N^{ϵ} -(carboxyethyl)lysine, pentosidine, and 3DG-imidazolone, or for OSPs and SRPs. Stainings for cytokeratin, a major protein component of MBs, and CML were co-localized. Furthermore, immunoblot analysis suggested that cytokeratin of MBs was modified to AGE, since a single protein band detected by a monoclonal anti-CML had a molecular weight identical to cytokeratin. The absence of the CML signal in MBs of hepatocellular carcinoma cells could be explained by scarce content of cytokeratin in carcinoma MBs.

Key words: advanced glycation end product; cytokeratin; immunohistochemistry; Mallory body; N^{ϵ} -(carboxymethyl)lysine

Abbreviations: ABC, avidin-biotin-immunoperoxidase complex; AEC, 3-amino-9-ethylcarbazole; AGE, advanced glycation end product; BSA, bovine serum albumin; CEL, N^{ϵ} -(carboxyethyl)lysine; CML, N^{ϵ} -(carboxymethyl)lysine; DAB, 3,3'-diaminobenzidine tetrahydrochloride; HCC, hepatocellular carcinoma; H&E staining, hematoxylin and eosin staining; HNE, 4-hydroxy-2-nonenal; MB, Mallory body; PAS, periodic acid Schiff; PBC, primary biliary cirrhosis; SDS, sodium dodecyl sulfate; SRP, stress-response protein

Mallory first described cytoplasmic hyaline degeneration in hepatocytes of alcoholic liver cirrhosis (Mallory, 1911). This cytoplasmic hyaline inclusion has been called Mallory body (MB). MB had been thought as specific to alcoholic liver disease (Edmondson, 1986). However, they are also associated with a number of non-alcoholic hepatobiliary diseases, such as Indian childhood cirrhosis, primary biliary cirrhosis (PBC), Wilson's disease, hepatocellular carcinoma (HCC) and adenomatous hyperplasia (Jensen and Gluud, 1994a; Terada et al., 1989). Although several different theories have been proposed for the formation of MBs (Jensen and Gluud, 1994b), the mechanism of MB formation as well as their developmental and pathological significance has remained unknown.

Ultrastructurally MBs consist of aggregates of filaments (Yokoo et al., 1972), and aberrant intermediate filaments of cytokeratin polypeptides (Katsuma et al., 1987). Recently, hyperphosphorylation of cytokeratin 8 and 18 (Stumptner et al., 2000) or ubiquitination of cytokeratin proteins was revealed in MB formation (Yuan et al., 1996). MBs were also known to be positive for α B-crystallin immunohistochemically (Lowe et al., 1992). Namely, MBs contain protein components such as cytokeratin, ubiquitin or α B-crystallin. Furthermore, the other chemical analyses demonstrated that MBs possess carbohydrates (Lyon and Christoffersen, 1971) and reducing sugar (Luisada-Opper et al., 1977) in addition to cytokeratin as the major protein component.

Long-term incubation of proteins with glucose leads, through the formation of early products such as Schiff base and Amadori rearrangement products, to the formation of advanced glycation end products (AGEs). Since monoclonal or polyclonal antibodies against these AGEs have been newly produced, immunological studies using anti-AGE antibodies have demonstrated accumulation of AGE-modified proteins in several human tissues in association with aging (Araki et al., 1992; Kimura et al., 1996) and several disorders such as diabetic complications (Makino et al., 1995), atherosclerosis (Kume et al., 1995) and

Alzheimer's disease (Smith et al., 1994). AGE-modification is known to occur in many proteins, especially long-lived proteins, which makes them insoluble, thus enhancing the deposition of modified proteins inside the cells or in the extracellular space (Makino et al., 1995; Giardino et al., 1996). Finally, AGE-modified proteins that are accumulated in the tissues exhibit direct toxic effects on cells biologically (Vlassara et al., 1994).

AGE-structures reported so far include fluorescent and cross-linking structures such as pentosidine (Sell and Monnier, 1990) and crossline (Nakamura et al., 1992), and nonfluorescent and non-cross-linked structures such as imidazolone (Niwa et al., 1997), N^ϵ -(carboxymethyl)lysine (CML) (Ahmed et al., 1986) and pyrrolidine (Hayase et al., 1989). The purpose of the present study was to investigate whether AGE-modification could play a role in MB formation. To do this end, human liver tissues with MBs were examined immunohistochemically using antibodies against CML, N^ϵ -(carboxyethyl)lysine (CEL), pyrrolidine, pentosidine and 3DG-imidazolone. Our results revealed that chemical modification of cytokeratin by CML, a major antigenic AGE-structure, is involved in MB formation in hepatocytes in alcoholic liver disease and primary biliary cirrhosis in contrast to no involvement of CML in MBs in HCC.

Materials and Methods

Patients

Eight hundred liver tissues from autopsy, biopsy and surgical files kept in our Department and Division were surveyed from 1973 to 2000, and MBs were found histologically in 80 cases (8 autopsy cases, 11 biopsy cases and 61 surgical cases). The 80 cases with MBs included three PBC cases (females, aged 37 to 68 years), 17 cases of alcoholic liver disease (15 males and 2 females, aged 33 to 66 years) and 60 cases of HCC (52 males and 8 females, aged 29 to 80 years). Surgical and autopsy specimens of normal liver tissues and other

Table 1. Sources of primary antibodies and dilutions

Antibody	Clonality	Clone	Dilution	Source (reference)
Anti-MB-component protein antibody				
Cytokeratin, broad	Monoclonal		Ready-to-use	Nichirei (Tokyo, Japan)
α B-crystallin	Polyclonal		1:250	J. E. Goldman (Iwaki et al., 1989)
Ubiquitin	Polyclonal		1:1000	S. H. Yen (Lee et al., 1989)
Anti-AGE antibody				
CML	Monoclonal	6D12	0.5 μ g/mL	S. Horiuchi (Ikeda et al., 1996)
	Monoclonal	CMS10	0.5 μ g/mL	Kumamoto Immunochem. Lab. (Kumamoto, Japan)
Pyrraline	Polyclonal		1.0 μ g/mL	S. Horiuchi (Hayase et al., 1989)
Pentosidine	Polyclonal		1.0 μ g/mL	S. Horiuchi (Miyata et al., 1996)
CEL	Monoclonal	KNH-30	0.6 μ g/mL	Kumamoto Immunochem. Lab. (Kumamoto, Japan)
3DG-imidazolone	Monoclonal	JNH-27	0.5 μ g/mL	Kumamoto Immunochem. Lab. (Kumamoto, Japan)
Anti-OSP antibody				
Acrolein	Monoclonal		0.5 μ g/mL	NOF (Tokyo, Japan)
4-HNE	Monoclonal		0.5 μ g/mL	JICA (Fukuroi, Japan)
Anti-SRP antibody				
SRP27	Monoclonal		Ready-to-use	BioGenex (San Rmon, CA)
SRP32	Monoclonal		1:200	Santa Cruz (Santa Cruz, CA)
SRP72	Monoclonal		1:500	Amersham (Buckinghamshire, United Kingdom)
SRP90	Monoclonal		1:5000	Affinity BioReagent (Neshanic Station, NJ)

AGE, advance glycation end product; CEL, N^{ϵ} -(carboxyethyl)lysine; CML, N^{ϵ} -(carboxymethyl)lysine; 4-HNE, 4-hydroxy-2-nonenal; MB, Mallory body; OSP, oxidative stress protein; SRP, stress response protein.

organ tissues from 10 individuals (7 males and 3 females; aged 19 to 68 years) were also examined as controls. The protocols were approved by the Ethics Committee in Tottori University Faculty of Medicine (No. 761).

Histology and immunohistochemistry

After fixation in 10% buffered formalin, the specimens were embedded in paraffin, cut into 4- μ m-thick sections and examined by light microscopy. Liver sections were stained by the following routine methods: hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), digestive PAS, silver and azan stainings. The distribution patterns of MBs were classified according to the method of Nakanuma and Ohta (1986). HCCs were graded into grades I, II and III according to the histological grading system of Edmondson and Steiner (1954). Serial sections were used for immunohistochemical analysis. The sources of primary antibodies and their dilutions used are listed in Table 1. Sections were deparaffinized and endog-

enous peroxidase activity was quenched for 30 min with 0.3% H_2O_2 . Sections were then washed in phosphate-buffered saline (PBS), pH 7.4. Normal sera isologous with each secondary antibody were used as blocking reagents. Sections were incubated with one of the primary antibodies or PBS for 18 h at 4°C. As positive controls for CML-immunostaining, atherosclerotic lesions in the wall of aortae from control individuals were used, biopsy specimens of diabetic kidney for pyrraline or pentosidine staining and atherosclerotic tissues also served as positive controls for acrolein and HNE stainings. Some sections were incubated with anti-CML antibody that had been preabsorbed with 1 mg/mL of CML-modified bovine serum albumin (BSA). With respect to the preabsorption test, some sections were incubated with anti-CML, anti-pyrraline, or anti-pentosidine antibody that had been preabsorbed with excess CML-, pyrraline- or pentosidine-modified BSA, respectively. Bound antibodies were visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using the appropriate Vectastain

ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dako, Glostrup, Denmark) as the final chromogen. For labeling multiple antigens in the same tissue section, normal and abnormal structures in H&E-stained sections were identified, mapped and photomicrographed. The H&E sections were then decolorized in 70% ethanol containing 1% HCl, after which the sections were rehydrated, quenched for 30 min with 0.3% H₂O₂, rinsed in PBS and incubated with the first primary antibody for 18 h at 4°C. Bound antibody was visualized by the ABC method using 3-amino-9-ethylcarbazole (AEC; Vector Laboratories) as the chromogen, yielding a red product. After photographing the immunoreactive structures, AEC sections were decolorized using 100% ethanol, rinsed twice in PBS and incubated for 60 min at room temperature with glycine-HCl buffer (pH 2.2) to elute the immunoreactive products. The completeness of the elution process was verified by obtaining a negative reaction after reapplication of the appropriate ABC kit including a secondary antibody and AEC on the eluted sections. The sections were subsequently incubated with the second primary antibody for 18 h at 4°C, and immunoreactivity was visualized by the ABC method using DAB as the chromogen. The proportion of positively-immunostained MBs was classified into five different categories; category – means negative staining, category +/- a few MBs were stained weakly, category + less than 10%, category ++ 10–50% and category +++ more than 50%.

Immunoblot analysis

This analysis was carried out on one fresh liver sample of a patient with PBC (Patient 2, a 61-year-old female) and on a liver tissue of age-matched normal individual (a 68-year-old female). In brief, specimens were homogenized in Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 2% sodium dodecyl sulfate (SDS), 25% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue and 62.5 mM Tris-HCl, pH 6.8. The sample was

heated at 100°C for 5 min. Soluble protein extracts from the samples were separated on a SDS-polyacrylamide gel (10%–20% gradient, Bio-Rad) and transferred by electroblotting onto Immobilon PVDF (Millipore, Bedford, MA). After blocking with 5% skimmed milk for 30 min at room temperature, the blots were incubated with anti-CML antibody (6D12) overnight at 4°C and visualized with the Vectastain ABC kit and DAB. Appropriate molecular weight markers (Bio-Rad) were included in each run.

Results

Histology and immunohistochemistry

Three cases clinically diagnosed as PBC showed histopathologically liver cirrhosis, biliary (Table 2). The histopathological findings of 17 cases clinically

Table 2. Clinicopathological findings of 20 cases of PBC and ALD with MBs

Case number	Age	Sex	Clinical diagnosis	Histopathology
1	37	F	PBC	LC/biliary
2	61	F	PBC	LC/biliary
3	68	F	PBC	LC/biliary
4	56	M	ALD	Fatty liver
5	44	M	ALD	AH
6	54	M	ALD	AH
7	47	M	ALD	AH
8	61	M	ALD	AH
9	37	M	ALD	Fibrosis
10	47	M	ALD	Fibrosis
11	49	F	ALD	Fibrosis
12	57	M	ALD	Fibrosis
13	65	M	ALD	Fibrosis
14	66	M	ALD	Fibrosis
15	37	F	ALD	LC/septal
16	56	M	ALD	LC/septal
17	59	M	ALD	LC/septal
18	70	M	ALD	LC/septal
19	33	M	ALD	LC/septal
20	61	M	ALD	LC/septal

AH, alcoholic hepatitis; ALD, alcoholic liver disease; F, female; LC/biliary, liver cirrhosis of biliary type (by Havana classification) (Sherlock, 1956); LC/septal, liver cirrhosis of septal type; M, male; MB, Mallory body; PBC, primary biliary cirrhosis.

cally diagnosed as alcoholic liver disease, showed fatty liver (1 case), alcoholic hepatitis (4 cases), liver fibrosis (6 cases) and liver cirrhosis (septal type, 6 cases) by routine stainings (Table 2). Sixty cases of HCCs were classified as the grade I (15 cases), the grade II (41 cases) and the grade III (4 cases). MBs were found predominantly in peripheral areas of regenerative nodules of liver cirrhosis in PBC (Fig. 1A) and alcoholic liver disease, and also in periportal areas of fibrotic alcoholic liver disease. MBs were scattered in fatty liver and in alcoholic hepatitis. MBs in HCCs were found in the cancer tissues (Fig. 1B). The ratio of MB-bearing hepatocytes to total hepatocytes varied from a few to ~10 percent in liver fibrosis, liver cirrhosis and HCCs. The distribution patterns of MBs of PBC were classified as diffuse type (2 cases) and sparse type (1 case). The patterns of MBs of alcoholic liver disease were classified as diffuse type (2 cases) and as sparse type (15 cases). The patterns of MBs of HCCs were as clustering type (21 cases), diffuse type (26 cases) and sparse type (13 cases) (Table 3).

The shape of MBs in PBC and alcoholic liver disease showed granular (Fig. 2A), staghorn, or circular pattern (Fig. 2D). In contrast, the shape of MBs in HCCs was irregular (Fig. 1B). MBs of alcoholic liver disease, PBC and HCCs were

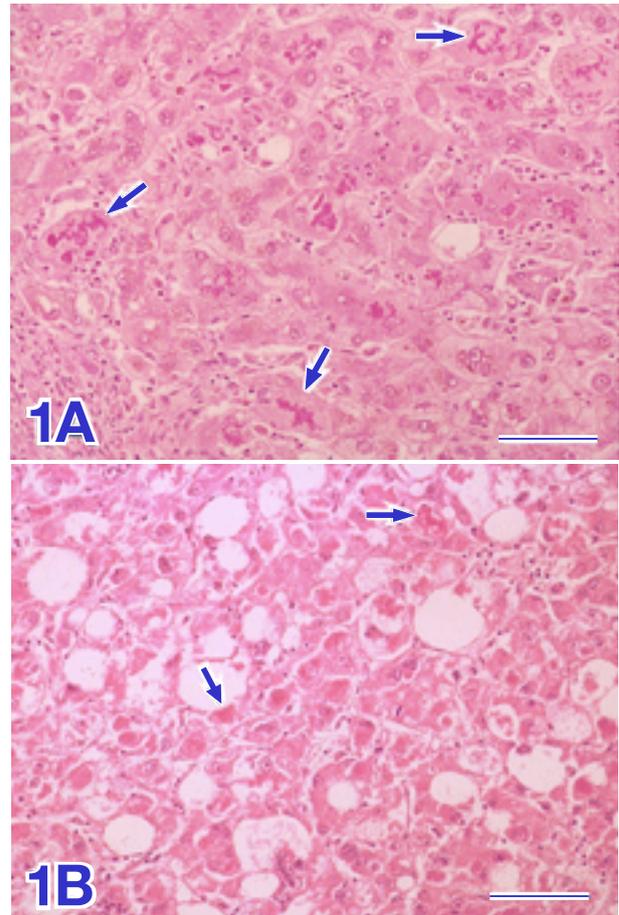


Fig. 1. Hematoxylin and eosin staining of liver tissue of Patient 2 with primary biliary cirrhosis (PBC) (A) and the patient with hepatocellular carcinoma (B), showing Mallory bodies (MBs) (arrows). Bar = 100 μ m.

Table 3. Histopathological and immunohistochemical findings of 80 cases of PBC, ALD and HCC with MBs

	Distribution pattern	Number of cases	CML (6D12)	CML (CMS10)	Pyrraline	Pentosidine	CEL	Cyto-keratin
PBC	Clustering	0						
	Diffuse	2	+~+++	+~+++	+/-~--	-	-	++
	Sparse	1	+	+	-	-	-	++
Alcoholic liver disease	Clustering	0						
	Diffuse	2	+~+++	+~+++	+/-	-	-	++
	Sparse	15	+	+	+/-~--	-	-	++
Hepatocellular carcinoma	Clustering	21						
	Diffuse	26	-	-	-	-	-	+/-
	Sparse	13	-	-	-	-	-	+/-

CEL, N^{ϵ} -(carboxyethyl)lysine; CML, N^{ϵ} -(carboxymethyl)lysine; MB, Mallory body; PBC, primary biliary cirrhosis. Distribution patterns of MBs were divided according to Nakanuma and Ohta (1986).

Proportion of MBs stained positively: +++, > 50%; ++, 10–50%; +, < 10%; +/-, only minor portion.