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De novo expression of nonhepatocellular cytokeratins in Mallory body formation

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Abstract Mallory bodies (MBs) are eosinophilic cytoplasmic inclusions observed predominantly in alcoholic liver disease. Although linked to disease activity, their pathogenesis is still unclear. Since intermediate filaments (cytokeratins) are major components of MBs, their cytokeratin polypeptide composition was analysed with monospecific antibodies for cytokeratins 7, 8, 14, 18, 19, and 20 by immunohistology. MBs were identified by light microscopy and ubiquitin immunostaining. All MBs were positive for cytokeratins 8 and 18. A significant percentage of the MBs was strongly positive for cytokeratins 19 and/or 20, which are not detectable in hepatocytes of normal liver and, in the case of cytokeratin 20, in hepatocytes of diseases devoid of MBs. MBs were essentially negative for cytokeratins 7 and 14. De novo expression of cytokeratins 19 and 20 was independent of the aetiology, occurring in all MB-associated diseases analysed, and seemed to precede MB formation, since in some hepatocytes a cytoskeletal-type staining pattern for these cytokeratins was present. In hepatocellular carcinomas cytokeratins 19 and 20 were frequently detected, but their cellular distribution was less closely associated with MBs. The ectopic expression of cytokeratins 19 and 20 appears to be related to MB formation and may take part in the derangement of the intermediate filaments during MB formation.

Key words Intermediate filaments · Alcoholic liver disease · Alcoholic hyalin

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Introduction

Mallory bodies (MBs) are unique eosinophilic condensations in the cytoplasm of hepatocytes. Since their first description in 1911 [19], they have remained one of the histological hallmarks of alcoholic liver disease [13]. Overall, MBs are most frequently found in alcoholic liver disease, but they are not specific for it and may occur in other chronic liver diseases, such as primary biliary cirrhosis (PBC) [26], Wilson's disease [31], Indian childhood cirrhosis [24] and hepatocellular carcinoma [23]. Their presence has been associated with a high rate of hepatocellular necrosis and activated inflammatory response, supporting the hypothesis that MBs may be indicators of disease activity. Experimentally, MB formation is induced in mice by griseofulvin intoxication [4]. Although MBs are found in severely damaged hepatocytes, these cells are not necessarily prone to death, since hepatocytes containing MBs are viable and MB formation may cease and revert after disappearance of the damaging agent [36].

MBs show a filamentous ultrastructure [7], representing aggregates of fimbriated rod-like structures that measure 14–20 nm in diameter [8]. Biochemically they reveal a mixed composition; their major constituents are ubiquitinated intermediate filament proteins [cytokeratins (CKs)], but they also contain unconjugated basic proteins, RNA, glycogen, phospholipids, and nonacid carbohydrate, while both actin [4] and tubulin [25] appear to be absent. A characteristic feature is their extremely low solubility, which may result from transglutaminase-mediated covalent crosslinks of MB proteins including CKs [38]. Based on the fact that MBs are mainly composed of cytokeratins, there are three major hypotheses about their origin.

The first is that MBs represent condensation products of pre-existing normal hepatocellular cytokeratins. This idea is based on the observation that in most cases reported by van Eyken et al. [33] and in the cases of Savolainen et al. [30], MBs were immunoreactive only for the hepatocellular cytokeratins CK 8 and CK 18, and also on the fact that the intermediate filament proteins in MB ag-

gregates appear to be linked to ubiquitin. Similarly, in griseofulvin-induced MBs of mice, hepatocellular cytokeratins A and D (corresponding to human CK 8 and CK 18) have been detected [5, 12].

According to the second hypothesis, MBs may be formed due to alterations in MB-associated proteins. MBs and Alzheimer's fibrils share many morphological and structural homologies and are both associated with a tau-phosphoprotein; it has been speculated that altered phosphorylation of tau-proteins is important in MB formation [16].

Finally, MB formation may be due to an altered CK expression profile. Since alcoholic liver disease is frequently accompanied by extensive ductular proliferation, investigators have analysed liver tissues with MBs for the presence of bile ductular cytokeratins that are normally not expressed in hepatocytes. Reactivity for cytokeratin polypeptides CK 19 and CK 7 has been described in some MBs and interpreted as evidence for a switch of hepatocytes towards a bile ductular epithelial phenotype during MB-related liver disease [15]. The presence of bile duct-epithelia-type CKs in MBs has also been suggested by Ray [29].

In this study we have analysed liver tissues from various diseases associated with MBs at stages before and after MB formation for the expression of potentially MB-associated CKs. Our analysis demonstrates that MBs are always strongly positive for CK 8 but negative for CKs 7 and 14, and many contain not only the bile duct-typical CK 19 but also CK 20, which is absent from normal liver [20, 21]. The positivity of MBs for CK 19 and/or CK 20 is found in all types of MB-associated liver diseases. De novo CK 19 and CK 20 expression in hepatocytes is MB associated but not disease specific and appears to precede MB formation in many cases.

Materials and methods

Biopsy materials and surgically excised liver tissues were fixed in 4% neutral buffered formalin and embedded in paraffin. For histology and immunohistology 6 µm sections were analysed. The histomorphology and immunohistology was characterized by at least two histopathologists independently. Analysis of the sections and photomicrography were performed with a Leica Diaplan microscope (Leica, Bensheim, Germany).

Immunohistological analysis was performed by the avidin–biotin complex (ABC) method, as previously described [3, 22]. The incubation conditions for the different cytokeratin antibodies (see Table 1) had been optimized using paraffin-embedded control tissues of known positive reactivity. For the detection of CK 7, antibody Ks 7.18 was used primarily; in selected cases, additional staining using antibody OV-TL 12/30 was performed. The specificity of the CK 20 immunostaining was corroborated by the use of four different monoclonal antibodies (Table 1; see [21]), which all yielded identical results. In some liver specimen stained for CK 20 using microwave pretreatment, hepatocytes showed weak granular cytoplasmic background staining, which was not observed us-

Table 1 Antibodies for cytokeratin expression analysis

Cytokeratin	Antibody (clone)	Incubation conditions for paraffin-embedded tissues	Control tissues	Supplier [reference]
7	Ks 7.18	Microwave; 0.001% trypsin; dilution 1:500	Liver (bile duct epithelia)	Progen Biotechnik, Heidelberg, Germany
7	OV-TL 12/30	Microwave; 0.001% trypsin; dilution 1:100	Liver (bile duct epithelia)	Sanbio BV, Uden, The Netherlands
8	CAM 5.2	Microwave; 0.001% trypsin; dilution 1:50	Liver tissue	Becton-Dickinson, Heidelberg, Germany
14	LL 001	Microwave; dilution 1:5	Skin	Cymbus Bioscience, Southhampton, UK [27]
18	Ks 18.04	Microwave; 0.001% trypsin	Liver tissue	Progen Biotechnik [5]
19	Ks 19.1	Microwave; 0.001% trypsin; dilution 1:10	Liver (bile duct epithelia)	Progen Biotechnik
20	IT-Ks 20.8	Microwave; 0.001% trypsin; dilution 1:200; or 0.1% trypsin; no microwave; dilution 1:10	Colon (mucosa)	Progen Biotechnik
20	IT-Ks 20.6	Microwave; 0.001% trypsin	Colon (mucosa)	Progen Biotechnik
20	IT-Ks 20.10	Microwave; 0.001% trypsin	Colon (mucosa)	Progen Biotechnik
20	IT-Ks 20.11	Microwave; 0.001% trypsin	Colon (mucosa)	Progen Biotechnik

Table 2 Hepatocellular cytokeratin expression in nonneoplastic conditions. (+ to +++ relative frequency of MBs, – no MBs present, *n.d.* not done, *neg.* negative, *pos.* expression, *numbers in brackets* estimated percentages of positive hepatocytes or MBs; when no percentage is given, semiquantitative evaluation was impossible due to low overall number of MBs)

Diagnosis	*n*	Case no.	MBs (relative frequency)	Cytokeratin expression in MB-free hepatocytes[a]				Cytokeratin expression in MBs		
				CK 7[b]	CK 14	CK 19	CK 20	CK 8[c]	CK 19	CK 20
Normal liver	10		–	neg.	neg.	neg.	neg.	–	–	–
Acute and chronic hepatitis	10		–	n.d.	neg.	n.d.	neg.	–	–	–
Alcoholic liver disease	19/	1	–	neg.	neg.	neg.	neg.	–	–	–
		2	–	neg.	neg.	neg.	neg.	–	–	–
		3	–	neg.	neg.	neg.	neg.	–	–	–
		4	–	neg.	neg.	neg.	neg.	–	–	–
		5	–	neg.	neg.	pos. (<1%)	pos. (<1%)	–	–	–
		6	+	n.d.	neg.	n.d.	pos. (<1%)	n.d.	n.d.	pos.
		7	+	neg.	neg. (<1%)	pos. (<1%)	pos.	pos. (100%)	pos. (10%)	pos.
		8	+++	neg.	neg.	pos. (<1%)	pos. (<1%)	pos. (100%)	pos. (40%)	pos. (10%)
		9	+ – ++	neg.	neg.	pos. (<1%)	neg.	pos. (100%)	pos. (60%)	pos. (20%)
		10	++	n.d.	neg.	pos. (2%)	pos. (<1%)	pos. (100%)	pos. (30%)	pos. (40%)
		11	++	n.d.	n.d.	pos. (<1%)	neg.	n.d.	pos. (80%)	pos. (80%)
		12	+	n.d.	n.d.	neg.	neg.	n.d.	pos.	neg.
		13	+	n.d. (OV-TL 20%)	n.d.	pos. (<1%)	pos. (2%)	n.d.	pos. (60%)	pos. (10%)
		14	+	n.d.	n.d.	pos. (<1%)	pos. (<1%)	n.d.	pos.	pos. (50%)
		15	+++	neg. (OV-TL 3%)	n.d.	neg.	neg.	pos. (100%)	neg.	pos. (3%)
		16	++	neg. (OV-TL 20%)	n.d.	pos. (1%)	neg.	pos. (100%)	neg.	neg.
		17	+++	neg. (OV-TL 10%)	n.d.	pos. (1%)	neg.	pos. (100%)	pos. (15%)	pos. (4%)
		18	–	neg. (OV-TL 20%)	n.d.	pos. (5%)	pos. (2%)	–	–	–
		19	+++	neg. (OV-TL 20%)	n.d.	pos. (<1%)	neg.	pos. (100%)	pos. (15%)	pos. (3%)
PBC	4/	1	+	n.d.	n.d.	pos. (<1%)	neg.	pos. (100%)	pos. (50%)	pos.
		2	+	neg.	n.d.	pos. (<1%)	neg.	pos. (100%)	pos. (50%)	pos. (10%)
		3	++	neg.	n.d.	pos. (<1%)	neg.	pos. (100%)	pos. (60%)	neg.
		4	+	neg.	n.d.	n.d.	neg.	pos. (100%)	n.d.	pos.
Wilson's disease	1		++	n.d.	n.d.	n.d.	neg.	n.d.	n.d.	pos. (40%)
PSC	1		+	neg.	n.d.	pos. (<1%)	neg.	pos. (100%)	pos. (80%)	pos.
Indian childhood cirrhosis	1		+++	neg. (OV-TL 5%)	neg.	pos. (2%)	pos. (2%)	pos. (100%)	pos. (10%)	pos. (20%)

[a] CK 8 and CK 18 were generally positive in hepatocytes in all cases tested
[b] As determined by antibody Ks 7.18; results for antibody OV-TL 12/30 are indicated in brackets
[c] For CK 18 similar results were obtained in four cases tested

ing the conventional technique with protease pretreatment (not shown), For help in the identification of MBs [18] in some of the cases of alcoholic liver disease, Indian childhood cirrhosis and hepatocellular carcinoma, immunohistology was performed with a rabbit polyclonal anti-bovine ubiquitin antibody (Dako), which cross-reacts with human ubiquitin, at a dilution of 1:150 (pretreatment of sections with 0.1% trypsin; no microwave heating).

For negative controls, which are particularly important in studies of dense proteinaceous material such as MBs, the primary antibody was replaced by phosphate-buffered saline. The monoclonal antibody against CK 14 also served as negative control, since all liver tissues of this study were completely negative. All negative controls yielded the expected negative results, including lack of staining in MBs.

Results

The results are summarized in Tables 2 and 3. Hepatocytes of both normal and diseased livers consistently showed positive immunostaining for CKs 8 and 18, whereas bile duct epithelium additionally expressed CKs 7 and 19. CK 20 was consistently absent in bile duct epithelia and hepatocytes of normal liver. In several cases of diseased liver very small proportions of (MB-free) hepatocytes exhibited diffuse cytoplasmic staining for CK 19 and CK 20 (Table 2; see also below), while CK 7 (antibody Ks 7.18) was negative in hepatocytes (Table 2). However, another monoclonal antibody against CK 7, OV-TL 12/30, stained up to 20% of hepatocytes of liver specimens with alcoholic liver disease (advanced fibrosis, cirrhosis; Table 2). These OV-TL 12/30-positive hepatocytes were seen predominantly but not exclusively in periportal and periseptal locations and often appeared in continuity with strong OV-TL 12/30-positive proliferated bile ducts (not shown). CK 14 was constantly negative.

Of the 19 specimens of alcoholic liver disease analysed by immunohistology for various cytokeratins (Tables 1, 2), 13 contained MBs. The MBs were identified by light microscopy and in selected cases (nos. 13, 15–20) by immunohistological staining with anti-ubiquitin antibodies (not shown). No specific signal was found for CK 14 in MBs. MBs were also negative for CK 7, with the exception of a single case (19) showing staining of occasional MBs with antibody OV-TL 12/30 (<1%) located in fields of CK 7-positive hepatocytes (see above). All MBs were strongly positive for CK 8; correspondingly, the antibodies against CK 8 and against ubiquitin, a general marker of MBs [18], stained similar numbers of MBs in serial sections. CK 18, a common complex partner of CK 8, also showed positive staining in MBs. A strong reactivity was found for CK 19 in a proportion of MBs (Fig. 1C, D). Surprisingly, reactivity for CK 20 was also found in MBs, but again only in a subpopulation of them (Fig. 1A, B). The pattern of CK 20 immunoreactivity was similar when four different monoclonal anti-human CK 20 antibodies that were directed against different epitopes of the molecule were employed (see Table 1). When staining patterns of CK 19 and 20 were compared in a case-by-case manner they were found to be partly overlapping, but not identical. All tissues with MBs appeared to contain some MBs positive for at least one of these two cytokeratins (see Table 2). Overall, it can be stated that minor but significant proportions of MBs exhibit strong immunoreactivity for either CK 19 or CK 20 or both, with CK 19-positive MBs usually exceeding CK-20-positive MBs in amount.

When present, the immunoreactivities for CK 19 and CK 20 were concentrated in the MBs themselves. In most

Table 3 Cytokeratin expression in HCCs (*n.d.* not done, *neg.* negative, *pos.* positive, numbers in brackets indicate estimated percentage of positive cytoplasmic inclusions of percentage of tumor cells; when no percentage is given, frequency could not be evaluated due to overall + – +++ relative frequency of cytoplasmic inclusions)

Case no.	Grade	Cytoplasmic inclusions	CKs in MBs/pale inclusions				CKs in MB-free tumor cells[b]	
			8/18	19	20	Other CKs[a]	19	20
1	G1–2	–	–	–	–	–	pos. (<1%)	pos. (<1%)
2	G2	–	–	–	–	–	pos. (<1%)	neg.
3	G1–2	–	–	–	–	–	neg.	neg.
4	G2–3	+++ (pale inclusions)	pos. (100%)	pos. (100%)	pos. (>80%)	14 neg.	pos. (>90%)	pos. (80%)
5	G2	+++ (MBs)	pos. (100%)	neg.	neg.	7,14 neg.	neg.	neg.
6	G3	+	n.d.	pos.	neg.	14 neg.	pos. (3%)	pos. (<1%)
7	G2–3	–	–	–	–	–	pos. (15%)	pos. (<1%)
8	Mostly fibrolamellar	+ (MBs)	n.d.	pos.	pos.	14 neg.	pos. (<1%)	pos. (15%)
9	G3	+ (MBs)	n.d.	neg.	pos. (20%)	7,14 neg.	pos. (5%)	pos. (10–20%)
10	G2	–	–	–	–		pos. (1–2%)	neg.
11	G2	+ (MBs)	n.d.	neg.	neg.	n.d.	neg.	neg.
12	G2	–	–	–	–	–	neg.	pos. (<1%)
13	G2	–	–	–	–	–	pos. (<1%)	pos. (<1%)
14	G4	–	–	–	–	–	pos. (<1%)	neg.
15	G2	+ (MBs)	n.d.	pos. (100%)	neg.	n.d.	pos. (90%)	neg.
16	G2	–	–	–	–	–	neg.	neg.
17	G2	+++ (MBs)	pos. (100%)	pos. (4%)	pos. (3%)	7 neg.	neg.	neg.

[a] CK 7 tested with antibody Ks 7.18
[b] CK 8 and CK 18 were generally positive in the cases tested

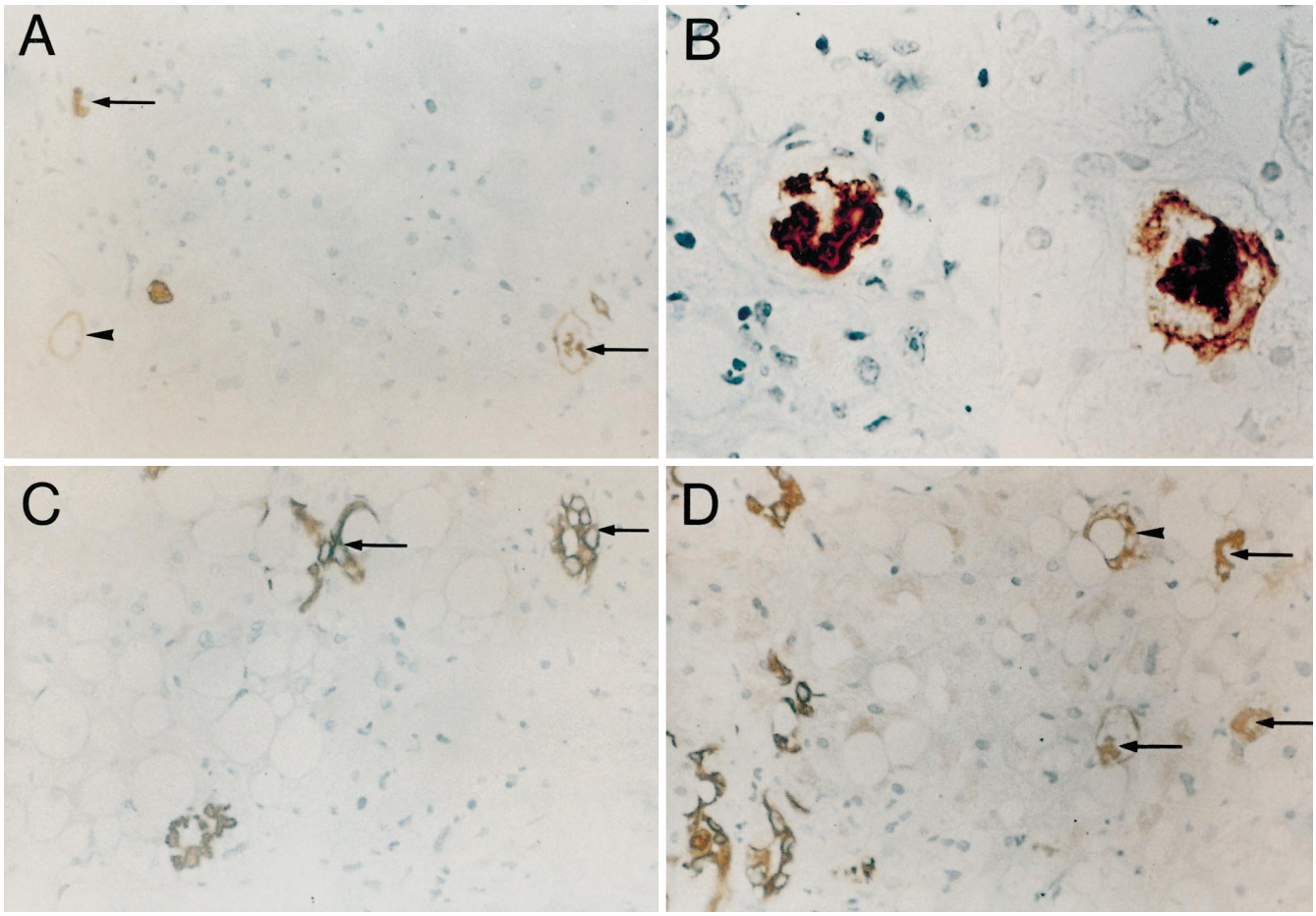


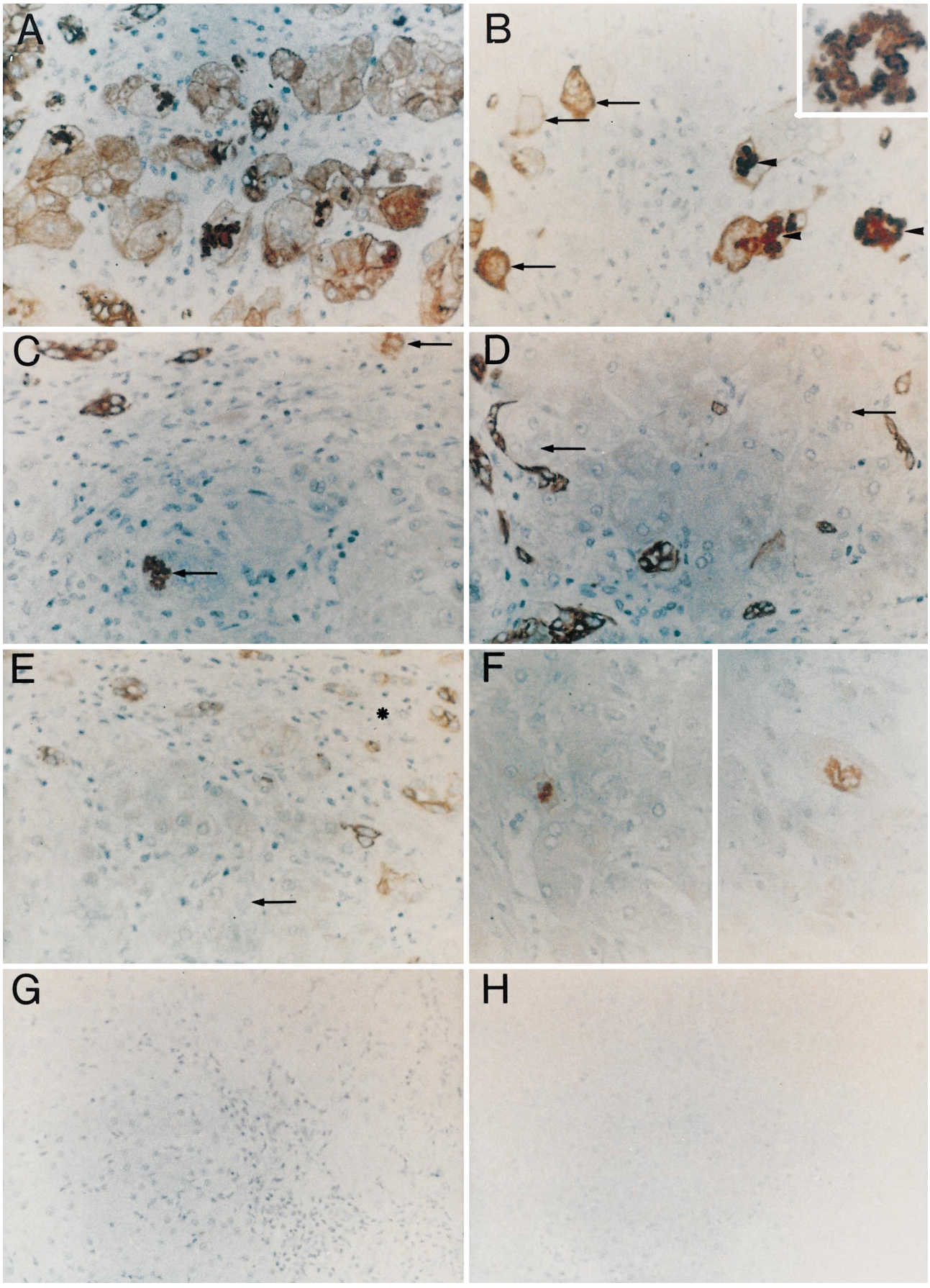
Fig. 1A–D Cytokeratin immunostaining in Mallory bodies (MBs) of alcoholic liver disease. **A** CK 20 is concentrated in MBs (*arrows*), but also found in a few hepatocytes devoid of MBs (*arrowhead*). **B** Single CK 20-positive hepatocytes from two different cases showing an almost exclusively MB-concentrated staining pattern (*left*), or a combination of MB-concentrated and cytoskeletal type of staining (*right*). **C** High CK 19 expression in an MB. *Arrows* depict bile ductules. **D** CK 19 is highly concentrated in MBs (*arrows*) and also found in an MB-free hepatocyte with a cytoskeletal type distribution (*arrowhead*); on the *left*, a fibrotic portal tract with bile ductules. **A**, **C**, **D** ×350, **B** ×800

cases, the remaining cytoplasm appeared negative, but MB-carrying hepatocytes were also detected that showed specific, lower or even strong cytoplasmic CK 19 or CK 20 positivity in a peripherally accentuated, cytoskeletal-type (CK filament type) staining pattern (Fig. 1B, right). Thus, in alcoholic liver disease MBs are frequently associated with hepatocellular neoexpression of CK 19 and/or CK 20 and the immunoreactivity for both cytokeratins is predominantly concentrated in the MBs themselves.

MBs are most frequently found in alcoholic liver disease, but are not specific for it. In order to determine whether CK 19 and CK 20 neoexpression was associated with MB formation in general or only present in alcoholic liver disease, we analysed a number of nonalcoholic liver diseases with MBs, namely 4 cases of PBC (Fig. 2F, left), 1 case of primary sclerosing cholangitis (PSC), 1 case of Wilson's disease (Fig. 2F, right), and 1 case of Indian childhood cirrhosis (Fig. 2A–D). In all of these cases the results were comparable to those observed in alcohol liver disease, showing variably frequent immunostaining for CK 19 and/or CK 20 in MBs (Fig. 2), while general MB staining was obtained with antibodies against CKs 8 and 18 and against ubiquitin (the last of which was analysed in Indian childhood cirrhosis only; not shown).

For control purposes 10 biopsy tissues without significant pathology and 10 tissues with acute or chronic hepatitis of various aetiology, all devoid of MBs, were incubated with CK 20 antibodies. In none of these tissues was hepatocellular reactivity found for CK 20 (Fig. 2G, H). Thus CK 19 and CK 20 neoexpression was related to MB formation, regardless of the underlying aetiology, but CK 20 was not detected in liver tissues with diseases not associated with formation MB.

During the analysis of CK 19 and CK 20 immunohistology in these cases positivity of hepatocytes was found to be mostly associated with the presence of MBs in the same cells, suggesting some kind of pathogenic relationship between these CKs and MBs. However, in several cases, some hepatocytes were detected that stained for CK 19 and/or CK 20 in a diffuse cytoplasmic pattern, but apparently did not contain MBs (Table 2). Furthermore, of 6 cases of alcoholic liver disease lacking MBs, 2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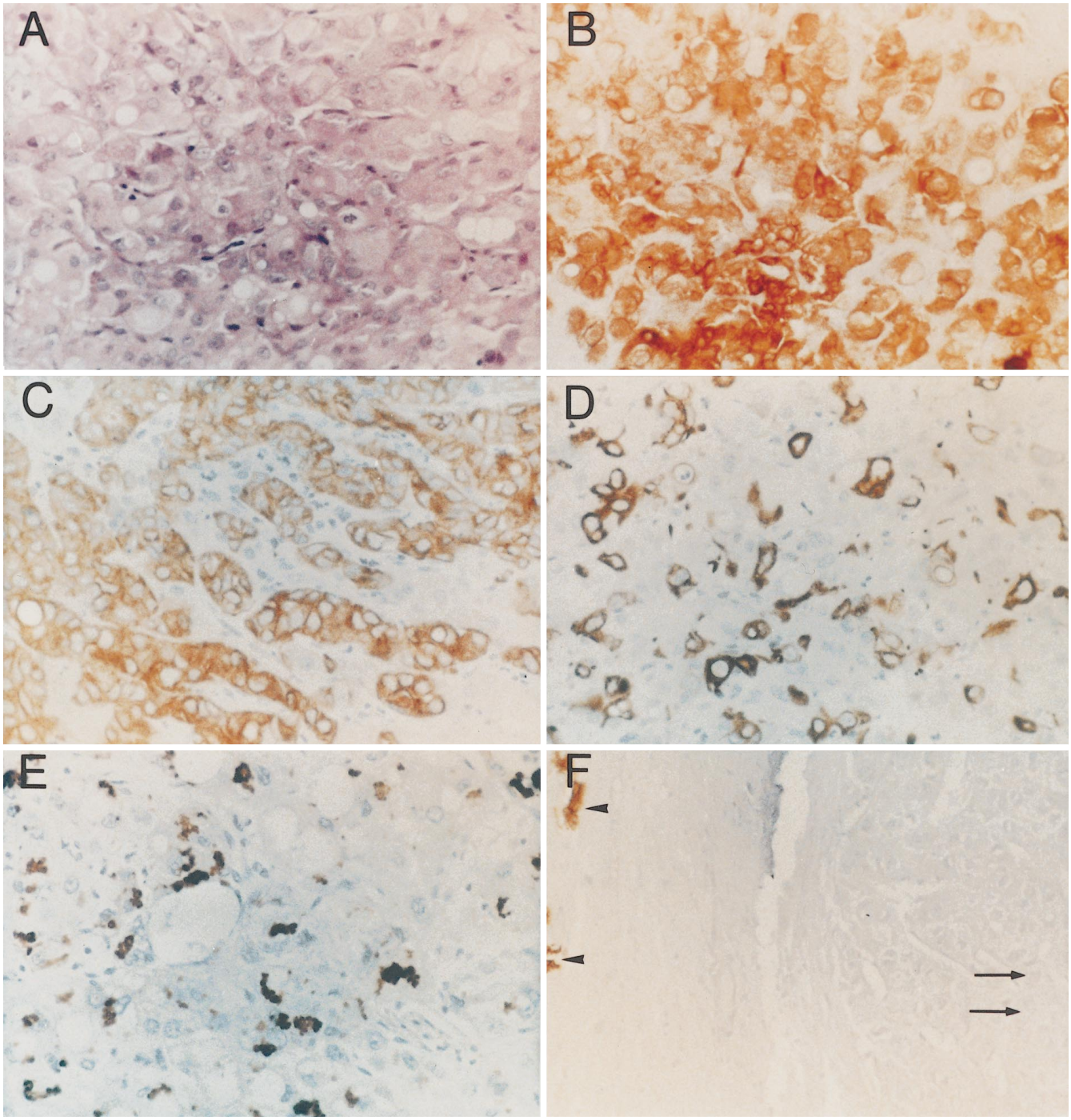

◀ **Fig. 2A–H** Cytokeratin expression in MBs of nonalcoholic liver disease. **A** CK 8 expression in a patient with Indian childhood cirrhosis; MBs are strongly accentuated. **B** CK 20 expression in MBs (*arrowheads*) and some damaged hepatocytes (*arrows*) in a patient with Indian childhood cirrhosis; *insert:* magnification of a CK 20-positive MB. **C** CK 19-positive MBs in a patient with Indian childhood cirrhosis (*arrows*). Note positive bile ductules in the *upper left corner.* **D** CK 19-negative MBs in a patient with Indian childhood cirrhosis (*arrows*). Note positive bile ductules on the *left.* **E** CK 7 expression in a patient with Indian childhood cirrhosis; MBs are negative (*arrow*), while bile ductules are positive (*asterisk*). **F** CK 20 expression in MBs of patients with primary biliary cirrhosis (*left*) and Wilson's disease (*right*). **G** No reactivity for CK 20 is detectable in a patient with chronic active hepatitis. **H** No CK 20 reactivity in liver tissue without significant pathology. **A**–**F** ×280, **G** insert in **B** ×800, **H** ×80

Fig. 3A–F Cytokeratin expression in hepatocellular carcinomas (HCCs) with cytoplasmic inclusions. **A** HCC no. 4 with numerous pale inclusions (H&E). **B** Almost all tumour cells of HCC 4 are positive for CK 19. **C** Area of homogeneous CK 20 positivity in HCC 4. **D** Area of mosaic CK 20 positivity in HCC 4. **E** Intense staining for CK 8 in MBs of tumour cells in HCC 5. **F** HCC 5 with absent reaction for CK 19 in MBs (*arrows* denote MBs, *arrowheads* denote bile ductules). **A**–**E** ×280, **F** ×160

showed diffuse cytoplasmic expression of CK 19 and CK 20 in a few hepatocytes (Table 2, cases 5 and 18). These findings suggest that CK 19 and CK 20 expression may precede MB formation.

Since MBs were also detected in a significant number of HCCs (6), we investigated a further 17 HCCs [6 with MBs, 1 with pale inclusions (Fig. 3A), 10 without significant cytoplasmic condensations] for cytokeratin expression (Table 3). The association of CK 19 or CK 20 with MBs was not as strict as that observed in the nonneoplastic cases. Although at the cellular level intense specific signals for CK 19 and CK 20 were sometimes detected in MBs, most MB-negative cases also exhibited at least a few tumour cells that were positive for CKs 19 and 20 with a diffuse or fibrillar cytoplasmic staining pattern. Two cases (5 and 11) with significant MB formation were negative for CK 19 (Fig. 3F) and CK 20, while the reactivity of the MBs for CK 8 was intense (Fig. 3E). In none of the cases was specific reactivity for CK 7 or CK 14 detectable. Furthermore, HCC 4 showed numerous pale inclusions that were strongly positive for CK 19 and CK 20; the immunostaining was, however, not restricted to the inclusions but mostly extended over the whole tumour cells (Fig. 3A–D).

Discussion

We have provided evidence that MB formation may be associated with de novo expression of the nonhepatocellular CKs 19 and 20, and that at least one of the two cytokeratins is present in the majority of cases of nonneoplastic liver diseases with MBs. This de novo expression most probably results from transcriptional activation, since none of the monospecific anti-CK 19 and anti-CK 20 antibodies employed shows cross-reactivity with polypeptide products of any of the other cytokeratin genes [14, 20, 21]. To our knowledge, this is the first study to show that the nonhepatic cytokeratin 20 can be a significant constituent of human MBs. CK 20 is a particular CK polypeptide expressed in only a small spectrum of epithelia, including gastric and intestinal epithelium, but absent from human fetal and adult liver [22]. Although biochemical confirmation is not yet available, we were able to corroborate the specificity of the immunostaining by the use of four independent monoclonal antibodies against CK 20. Interestingly, in nonneoplastic liver parenchyma, CK 20 seems to be restricted to MBs and single hepatocytes of MB-containing liver specimens, suggesting a high degree of correlation between CK 20 expression and MB formation. However, it is evident that CK 19 or CK 20 neoexpression alone is not a common denominator of MB formation, since CK 19 and CK 20 containing MBs do not add up to the total number of MBs in most analysed cases.

Nevertheless, there are several reasons to believe that hepatocellular activation of CK 19 and CK 20 genes may be a part of the spectrum of changes leading to intermediate filament derangement in MB formation and thus may take part in their formal pathogenesis. First, MBs contain cytokeratins as their major constituents [5, 36, 37], and CK 19 and/or CK 20 have been found in almost all cases of nonneoplastic liver disease with MBs. Moreover, expression of CK 19 and CK 20 in hepatocytes was mostly associated with the presence of MBs in the same cells, the immunoreactivity being concentrated mainly (but not exclusively) in the MBs themselves. Since de novo expression of CK 19 and/or CK 20 was observed in several MB-associated liver diseases of different aetiology, but not in liver tissues without MB formation, including normal liver as well as acute and chronic hepatitis, both events (CK 19/CK 20 expression and MB formation) seem to be linked pathogenetically.

Secondly, the presence of CK 19 and CK 20 expression in some MB-negative hepatocytes of liver tissues otherwise containing MBs and a few hepatocytes in a case with significant alcoholic liver disease lacking MBs suggests that de novo expression of CK 19 and/or CK 20 may precede MB formation. Thus, de novo expression of CK 19 and CK 20 is not simply a direct consequence of MB formation, but also seems to be connected with hepatocellular dysfunction prior to MB formation.

There is currently no significant evidence for a mechanism by which ectopically expressed CK 19 and/or CK 20 may contribute to MB formation, but a mechanistic speculation is that addition of high amounts of these type I CKs to the pre-existing CK 8 (type II) and CK 18 (type I) may result in a disturbed type I/type II stoichiometry, which may lead to increased proteolysis [17] and perhaps facilitate crosslinking of CKs and associated cellular proteins in hepatocytes. The basis for this hypothesis is the fact that normal CK filaments are composed of equimolar amounts of type I and type II CKs, the basic structural subunit being a heterodimer [9, 11]. Excess CK molecules of only a single type would not be able to assemble into typical intermediate filaments. (It should be noted in this context that MB filaments are not identical with intermediate filaments [8]). Altered stoichiometry of CK proteins in MBs has also been suggested previously [29, 37]; however, proof is still lacking. Because of the high degree of covalent protein crosslinks in MBs [38], protein biochemical analyses of MBs are extremely difficult. In fact, even in strong lysis buffers, most material of MBs remains insoluble, and most of the material eventually solubilized and loaded onto polyacrylamide gels does not enter the gels during electrophoresis or remains at the interphase between stacking and dissolving gel [37]. Moreover, immunohistochemistry alone does not allow sound quantitative conclusions. Therefore our hypothesis of possible CK imbalance due to excess amounts of type I CKs currently remains speculative. Also, the finding that some MBs lacked both CK 19 and CK 20 indicates that these proteins are not required for MB formation; conversely, the presence of CK 19 and CK 20 in hepatocytes does not necessarily indicate MB formation. Interestingly, forced expression of only a single CK polypeptide in NIH3T3 cells does not lead to the formation of filamentous structures but to granular cytoplasmic aggregates up

to 1 μm in diameter [6]. Furthermore, recent experimental data indicate that forced overexpression of CK 8 or CK 18 alone in tumour cell lines leads to abnormal cytoplasmic CK aggregates, while cotransfection of both cytokeratins yields normal CK filaments [32]. Whether this phenomenon correlates with the up to 7-fold overexpression of CK-mRNA in experimental models of MB formation [10] is currently unknown.

In some hepatocytes a cytoskeletal-type staining pattern for CK 20 and CK 19 was present beside positive MBs. This may suggest that newly synthesized CK 20 and CK 19, when accompanied by appropriately synthesized type I CK 8, may first (totally or partly) be assembled into a normal intermediate filament network, which subsequently may become rearranged into MBs. Moreover, out data on hepatocellular carcinomas show that CK 19 and CK 20 may exist without association with MBs but most probably as regular intermediate filaments. False incorporation of the hepatocellular CKs may also be a process that potentially promotes MB formation. Precipitation or resorption of the existing intermediate filaments by the introduction of 'false' or inappropriately expressed intermediate filament proteins is generally believed to be a degeneration-associated phenomenon occurring in cell types as diverse as myogenic cells, squamous epithelial of the skin and neurons [35]. Expression of a truncated cytokeratin leads to the disruption of the intermediate filament network of epidermal cells in vitro and in vivo [2, 34].

Owing to the previously reported CK 19 expression in MB-carrying hepatocytes, it has been suggested that MB formation may be the consequence of a switch of hepatocytes towards a bile duct-like phenotype [15]. Results presented in this paper argue against this assumption, since MB-carrying hepatocytes can express CK 20, which is not present in normal biliary epithelium, and do not resemble biliary epithelial cells by any morphological characteristics. Furthermore, although alcohol-damaged hepatocytes may switch on expression of the bile-duct-typical CK 7 ([33] and present results with antibody OV-TL 12/30), most MBs were negative with the antibodies against this CK employed. MBs were also negative for CK 14. This does not, however, fully exclude the possibilities that CK 7 and/or CK 14 might also be MB components, since antigenic epitopes could be masked.

In normal liver both of the CK 7 antibodies we used strongly stained bile ducts only, but conspicuous discrepancies were found in diseased liver in that OV-TL 12/30 but not Ks 7.18 stained subpopulations of hepatocytes and very occasionally also MBs. Both antibodies have been well characterized at the protein level by Western blotting, showing specific reactivity for CK 7 [1, 28], but OV-TL 12/30 displayed a broader reactivity pattern in several normal tissues and carcinomas as compared to other established CK 7 antibodies [28]. These discrepancies may be due to epitope masking under certain conditions including conformational alteration of CK 7 in certain cell types or to higher sensitivity of OV-TL 12/30, then implying underrepresentation of CK 7 staining for all CK 7 antibodies except OV-TL 12/30. In contrast OV-TL 12/30 may crossreact with another yet unknown protein, giving an additional false-positive reactivity of the antibody.

It is interesting to note that CK 20 neoexpression in nonneoplastic hepatocytes is relatively closely associated with the MB formation in the context of a distinct spectrum of liver damage, whereas in neoplastic hepatocytes of hepatocellular carcinomas it is a more widespread phenomenon, less clearly coupled with MBs. The present data confirm and extend our previous observations that the focal expression of CK 20 is a fairly typical feature of the CK pattern of hepatocellular carcinomas [21]. The ability of hepatocytes to switch on the expression of CK 20, which is typical of some endodermal tissues [20], may appear reasonable in view of the endodermal embryonic derivation of the liver. Interestingly, in the rat, bile ducts express CK 20 [20].

Although altered expression of CKs, including CK 19 and CK 20 neoexpression, may be relevant for MB formation, it is unlikely to be the only relevant pathogenetic process. A tau-protein with altered phosphorylation is associated with MBs and thus represents an analogy with neurofibrillary tangles of Alzheimer's disease [16]. Since this process is unlikely to lead to transcriptional activation of CK 19 or CK 20, it will have to be tested whether both events are independent or whether altered tau-phosphorylation can be a consequence of de novo CK 19 or CK 20 expression. Recently it has been reported that after rechallenge of griseofulvin-treated mice CKs appeared in MBs prior to phosphorylated tau [10].

Currently it is not possible to conclude that transcriptional activation of CK genes is the initial impetus that finally leads to MB formation. Nevertheless, it seems to be one of several important events ultimately leading to cytokeratin intermediate filament derangement (and thus MB formation). The present data contribute to the characterization of molecular events in MB-associated hepatocellular damage that eventually should successfully identify the molecular convergence of the damaging processes that occur during different hepatic diseases.

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References

1. Bartek J, Vojtesek B, Staskova Z, Batkova J, Kerekes Z, Reijthar A, Kovarik J (1991) A series of 14 new monoclonal antibodies to keratins: Characterization and value in diagnostic histopathology. J Pathol (Lond) 164:215–224
2. Coulombe P, Hutton M, Vassar R, Fuchs E (1991) A function for keratins and a common thread among different types of epidermolysis bullosa simplex diseases. J Cell Biol 115: 1661–1674

3. Demirkesen C, Hoede N, Moll R (1995) Epithelial markers and differentiation in adnexal neoplasms of the skin: an immunohistochemical study including individual cytokeratins. J Cutan Pathol 22:518–535
4. Denk H, Franke WW, Eckerstorfer R (1979) Formation and involution of mallory bodies (alcoholic hyalin) in murine and human liver revealed by immunofluorescence microscopy with antibodies to prekeratin. Proc Natl Acad Sci USA 76:4112–4116
5. Denk H, Krepler R, Lackinger E, Artlieb U, Franke WW (1982) Immunological and biochemical characterization of the keratin-related component of Mallory bodies: a pathological pattern of hepatocytic keratins. Liver 165–175
6. Domedjoud L, Jorcano JL, Breuer B, Alonso A (1988) Synthesis and fate of keratins 8 and 18 in nonepithelial cells transfected with cDNA. Exp Cell Res 179:352–361
7. Flax MH, Tisdale WA (1964) An electron microscopic study of alcoholic hyalin. Am J Pathol 44:441–453
8. Franke WW, Schmid E, Kartenbeck J, Mayer D, Hacker H-J, Bannasch P, Osborn M (1979) Characterization of the intermediate-sized filaments in liver cells by immunofluorescence and electron microscopy. Biol Cell 34:99
9. Fuchs E, Weber K (1994) Intermediate filaments: structure, dynamics, function, and disease. Annu Rev Biochem 63:345–382
10. Fuchsbichler A, Stumptner C, Lehner M, Denk H, Zatloukal K (1996) Disturbance of microtubules and overexpression of cytokeratin in hepatocytes are key events in mallory body formation (abstract) 11th European Cytoskeleton Forum, Maastricht 31.8.–4.9 1996, book of abstracts, p33
11. Hatzfeld M, Franke WW (1985) Pair formation and promiscuity of cytokeratins: Formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombinations of purified polypeptides. J Cell Biol 101:1826–1841
12. Hazan R, Denk H, Franke WW, Lackinger F, Schiller DL (1986) Change of cytokeratin organization during development of Mallory bodies as revealed by a monoclonal antibody. Lab Invest 64:543–553
13. Jensen K, Gluud C (1994) The Mallory body: morphological, clinical and experimental studies (part 1 of a literature survey). Hepatology 20:1061–1077
14. Kasper M, Stosiek P, Typlt H, Karstens U (1987) Histological evaluation of three new monoclonal anti-cytokeratin antibodies. I. Normal tissues. Eur J Clin Oncol 23:137–147
15. Katsuma Y, Swierenga SHH, Ketthry U (1987) Changes in the cytokeratin intermediate filament cytoskeleton associated with Mallory body formation in mouse and human liver. Hepatology 7:1215–1223
16. Kenner L, El-Shabrawi Y, Hutter H, Forstner M, Zatloukal K, Hoefler G, Preisegger KH, Kurzbauer H, Denk H (1994) Expression of three- and four-repeat Tau isoforms in mouse liver. Hepatology 20:1086–1089
17. Knapp A, Franke WW (1989) Spontaneous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occurring at different levels of regulation. Cell 59: 67–79
18. Lowe J, Blanchard A, Morrell K, Lennox G, Reynolds L, Billett M (1988) Ubiquitin is a common factor in intermediate filament inclusion bodies of diverse type in man, including those of Parkinson's disease, Pick's disease, and Alzheimer's disease as well as Rosenthal fibres in cerebellar astrocytomas, cytoplasmic bodies in muscle, and Mallory bodies in Alcoholic liver disease. J Pathol 155:9–15
19. Mallory FB (1991) Cirrhosis of the liver: five different types of lesions from which it may arise. Bull Johns Hopkins Hosp 22:69–75
20. Moll R, Schiller DL, Franke WW (1990) Identification of protein IT of the intestinal cytoskeleton as a novel type I cytokeratin with unusual properties and expression patterns. J Cell Biol 111:567–580
21. Moll R, Löwe A, Laufer J, Franke WW (1992) Cytokeratin 20 in human carcinomas. A new diagnostic marker detected by monoclonal antibodies. Am J Pathol 140:427–447
22. Moll R, Zimbelmann R, Goldschmidt MD, Keith M, Laufer J, Kasper M, Koch PJ, Franke WW (1993) The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation 53: 75–93
23. Nakanuma Y, Ohta G (1986) Expression of Mallory bodies in hepatocellular carcinoma in man and its significance. Cancer 57:81–86
24. Nayak NC, Sagreiya K, Ramalingaswami V (1969) Indian childhood cirrhosis: the nature and significance of cytoplasmic hyalin in hepatocytes. Arch Pathol 88:631–637
25. Ohta M, Marceau N, Perry G, Manetto V, Gambetti P, Autilio-Gambetti L, Metuzals J (1988) Ubiquitin is present on the cytokeratin intermediate filaments and Mallory bodies of hepatocytes. Lab Invest 59:848–856
26. Portmann B, Popper H, Neuberger J, Williams R (1985) Sequential and diagnostic features in primary biliary cirrhosis based on serial histologic study in 209 patients. Gastroenterology 88:1777–1790
27. Purkis PE, Steel JB, Mackenzie IC, Nathrath WBJ, Leigh IM, Lane EB (1990) Antibody markers of basal cells in complex epithelia. J Cell Sci 97:39–50
28. Ramaekers F, van Niekerk C, Poels L, Schaafsma E, Huijsmans A, Robben H, Schaart G, Vooijs P (1990) Use of monoclonal antibodies to keratin 7 in the differential diagnosis of adenocarcinomas. Am J Pathol 136:641–655
29. Ray MB (1987) Distribution patterns of cytokeratin antigen determinants in alcoholic and non-alcoholic liver diseases. Hum Pathol 18:61–66
30. Savolainen VT, Lalu K, Penttilä A, Virtanen I, Karhunen PJ (1994) Cytokeratin inclusions in alcoholic liver disease and their relation to the amount of alcohol intake. Liver 14: 281–287
31. Stromeyer FW, Ishak KG (1980) Histology of the liver in Wilson's disease: a study of 34 cases. Am J Clin Pathol 77:12–24
32. Stumptner C, Fuchsbichler A, Denk H, Zatloukal K (1996) Overexpression of cytokeratin induces accumulation of the Malloy body-specific component M_M120–1 (abstract) 11th European Cytoskeleton Forum, Maastricht, 31.8.–4.9. 1996, book of abstracts, p 68
33. Van Eyken P, Sciot R, Desmet VJ (1988) A cytokeratin immunohistochemical study of alcoholic liver disease: evidence that hepatocytes can express "bile duct-type" cytokeratins. Histopathology 13:605–617
34. Vassar R, Coulombe P, Degenstein L, Albes K, Fuchs E (1991) Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. Cell 64:365–380
35. Yu KR, Hijikata T, Lin ZX, Sweeney HL, Englander SW, Holtzer H (1994) Truncated desmin in PtK2 cells induces desmin-vimentin-cytokeratin coprecipitation, involution of intermediate filament networks, and nuclear fragmentation: a model for many degenerative diseases. Proc Natl Acad Sci USA 91: 2497–2501
36. Zatloukal K, Spurej G, Rainier I, Lackinger E, Denk H (1990) Fate of Mallory body-containing hepatocytes: disappearance of Mallory bodies and restoration of the hepatocytic intermediate filament cytoskeleton after drug withdrawal in the griseofulvin-treated mouse. Hepatology 11:652–661
37. Zatloukal K, Böck G, Rainer I, Denk H, Weber K (1991) High molecular weight components are main constituents of mallory bodies isolated with a fluorescent activated cell sorter. Lab Invest 64:200–206
38. Zatloukal K, Feus L, Denk H, Tarcsa E, Spurej G, Böck G (1992) High amount of ε- (γ-glutamyl) lysine cross-links in Mallory bodies. Lab Invest 66:774–777