MBs were not stained with antibodies against oxidative stress proteins (OSPs) such as acrolein and HNE nor against stress response proteins (SRPs) (27 kDa, 32 kDa, 72 kDa or 90 kDa) (data not shown). MBs in HCCs were also identified by positive staining to anti-ubiquitin antibody (Fig. 3A). MBs in all cases of HCCs were weakly stained by anti-cytokeratin antibody. By contrast, MBs in HCCs were not stained to CML (Fig. 3B), pyrraline, pentosidine, CEL, 3DG-imidazolone, two types of OSPs (acrolein and HNE) nor four types of SRPs (srp27, 32, 72 and 90). There were no significant differences in AGE-expressions among the histologically different specimens in HCCs.

The proportion of positively-immunostained MBs varied from one sample to another, ranging from less than 10% to more than 50% of total MBs (Table 3). Immunohistochemical stainings of CML of PBC and alcoholic liver disease showed that 1 out of 20 cases was of category +++; 4 out of 20 cases were of category ++, and 15 cases out of 20 cases were of category +. Immunohistochemical stainings of pyrraline of PBC and alcoholic liver disease showed that one case of PBC was of category +/– and nine of 17 cases of alcoholic liver disease were of category +/–. MBs with diffuse distribution pattern were positive for CML or pyrraline more than MBs with sparse type. All cases of PBC and alcoholic liver disease were of category ++ about cytokeratin. All cases of HCC were of category +/– about cytokeratin.

Hepatocytes from 10 control individuals were not stained by five anti-AGE antibodies against either of CML, pyrraline, pentosidine, CEL or 3DG-imidazolone. Similarly, normal hepatocytes showed no immunoreactivities to OSPs, SRPs, αB-crystallin nor ubiquitin. No staining was detected when sections were incubated with PBS. The specificity and high affinity of these antibodies were confirmed by control tissues. As expected (Makino et al., 1995; Kume et al., 1995; Horie et al., 1997), CML-immunoreactivities were observed in smooth muscle cells of atherosclerotic lesions, and immunoreactivities for pyrraline, pentosidine, CEL and 3DG-imidazolone were also noted in the thickening intimae of arteries. As reported earlier (Uchida et al., 1995), macrophage-derived foam cells in the atheromatous lesions were positive for HNE. Anti-CML antibody pretreated with an excess amount of CML-modified BSA did not stain smooth muscle cells in the atherosclerotic lesions. Similarly, anti-pyrraline, or anti-pentosidine antibody pretreated with an excess amount of pyrraline- or pentosidine-modified BSA did not stain the thickening intimae of arteries.

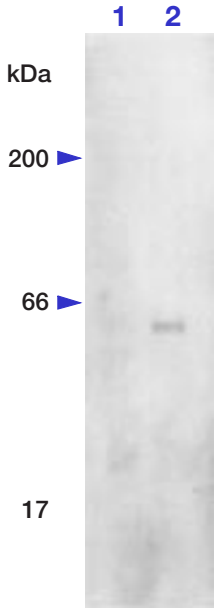

Fig. 4. Western blot analysis using monoclonal CML (with monoclonal antibody 6D12) in liver tissues.

Lane 1: normal control liver.
Lane 2: primary biliary cirrhosis liver (Patient 2).

Immunoblot analysis

The results of immunoblot analyses are shown in Fig. 4. When the liver-tissue homogenate of PBC (Patient 2, a 61-year-old female), whose hepatocytes were demonstrated to contain CML-positive MBs immunochemically, was subjected to immunoblotting with anti-CML antibody, a single band with a molecular weight indistinguishable from that of cytokeratin was detected (Fig. 4). Immunoblotting of the fresh autopsy liver specimen of a normal individual (a 68-year-old female) did not show any specific band (Fig. 4).

Discussion

Although the frequency and the distribution of MBs that were detected by H&E staining were various among 80 cases examined, all of the MBs were positive for ubiquitin, αB-crystallin and cytokeratin immunohistochemically. MBs in HCCs were weakly positive for cytokeratin, but MBs in PBC and alcoholic liver disease were strongly positive for cytokeratin. The present immunohistochemical results coincide with the previous reports which demonstrated that MBs consisted of aggregates of cytokeratin filaments (Yokoo et al., 1972) or that cytokeratin protein of MBs was ubiquitinated (Yuan et al., 1996). In the present study, the facts that MBs contain cytokeratin, ubiquitin and αB-crystallin as protein components were confirmed immunohistochemically.

The novel finding of the present study is that MBs in PBC and alcoholic liver disease were positive for CML and pyrraline in contrast to those of HCCs. In cases of PBC and alcoholic liver disease, immunohistochemical results of the same paraffin sections using anti-CML and anti-cytokeratin antibodies, revealed that both CML and cytokeratin were co-localized on the same MBs. These results suggest that MBs in PBC and alcoholic liver disease have epitopes of CML addition to cytokeratin. No CML-positive reactivities except MBs were found in the liver sections of PBC, alcoholic liver disease, HCCs and normal controls. Furthermore, immunoblot analysis with anti-CML antibody supported the immunohistochemical findings. Single band with a molecular weight about 55 kDa was detected in the liver-tissue homogenate of PBC. This result demonstrated that liver tissue-homogenate of PBC contain CML-combined protein with molecular weight about 55 kDa. Considering the fact that the molecular weight of ubiquitin, one of protein components of MBs is about 8 kDa (Lee et al., 1989; Lowe and Mayer, 1990) and that of αB-crystallin is also 22 kDa (Iwaki et al., 1989), it was suggested that CML-combined protein might be cytokeratin.

Glycation is one of biochemical reactions and it occurs when proteins were incubated with reducing sugars. Finally, CML, CEL or pyrraline-combined proteins through several steps by glycation form AGEs. Although oxidation is necessary for the formation of AGEs in vitro (Nagai et al., 1997), the intention level of oxidation, the nature of oxidative processes, and the period of oxidative stresses are of different in vivo. At the cellular level, living cells can induce a diverse group of SRPs in response to different types of biological stresses, including oxidative damage (Morimoto et al., 1990). Since OSPs and SRPs in MBs were not detected in the present immunohistochemical studies, the amounts of these compounds in MBs might be, if any, negligible, suggesting that the oxidative stress that generates these marker compounds does not contribute to the process of MB formation in vivo.

Modification by glycation occurs in many proteins in relation to the pathogenesis of diseases such as atherosclerosis (Kume et al., 1995), diabetic complications (Makino et al., 1995), Alzheimers' disease (Smith et al., 1994). Furthermore, AGEs are common to be long-lived, insoluble molecules, readily deposited in cells that have a direct cytotoxic effect (Vlassara et al., 1994). Although several hypotheses of MBs have been discussed (Jensen and Gluud, 1994a, 1994b) the results of the present study clarified that AGE-modification of cytokeratin, a major protein component of MBs, plays an important role in the formation of MBs in hepatocytes in the PBC and the alcoholic liver disease. Taken together with abnormal cytokeratin aggregation toxicity, it is conceivable that the AGE modification of cytokeratin in MBs could amplify the aggregation of cytokeratin and that the formation of the AGEs could result in greater toxicity in hepatocytes-bearing MBs in patients with the PBC and alcoholic liver disease. Considering the facts that MBs of HCCs contain less amount of cytokeratin protein, HCC cells form MBs for a short disease duration of tu-

morigenesis or have abnormal biological metabolism, AGE-modification do not contribute to the formation of the MBs in HCC cells. To elucidate the differences between AGE-expressions of alcoholic liver disease or PBC and AGE-expressions of HCCs, a further complete understanding of the molecular mechanisms of MB formation in hepatocytes will be necessary. Our results suggest that the formation of AGEs might be cytotoxic to MB-containing hepatocytes in PBC and alcoholic liver disease in contrast to HCCs.

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