Expression of Cytokeratins 8 and 18 on Mallory Body and Oval Cell in Rats during Hepatic Fibrosis

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Abstract. *Background: The aim of this study was to determine the induction and distribution of Mallory body (MB) and oval cells in carbon-tetrachloride (CCl₄)-induced rat liver fibrosis. Materials and Methods: MBs and oval cells expressing cytokeratins (CKs) 8 and 18 were monitored by immuno-histochemistry and immunoblotting. Results: MBs were mainly detected within hepatocytes near the fibrotic areas, and oval cells were located along or in the fibrotic areas. Both MBs and oval cells increased in size and number in the development of fibrosis. At cirrhotic liver, most of the oval cells were located in the fibrous septa and around newly formed bile ductules. Moreover, as hepatic injuries developed into fibrosis, a much more prominent single band of CK18 was detected. Conclusion: The occurrence and distribution of MB and oval cells in CCl₄-induced rat liver fibrosis are reported. This represents the first CCl₄ experimental in vivo model of MB induction, which will be useful for further investigations on the pathogenesis of MB.*

Cytokeratins (CKs) form cytoplasmic intermediate filament networks in most epithelial cell types and have been considered as rather static structures, primarily responsible for mechanical cell stability (1-4). The CK family is composed of at least 20 members, designated as CK1 to CK20, whose expression patterns differ among different epithelial tissues (5). In normal adult liver, hepatocytes contain only CK8 and CK18, which are distributed as a cytoplasmic filament network and as a band just below the microfilaments surrounding the plasma membrane (6, 7). Recent reports have demonstrated that CK8 and CK18 were identified as major Mallory body (MB) components in humans and experimental animals (1, 8-10), and some have suggested that MBs were aggresomes of CK8 and CK18 (11, 12). For many years MBs were characteristically observed in alcoholic liver disease, but it was accepted that MBs were not specific for this disease and may occur in other non-alcoholic and chronic liver diseases of experimental animal models (1-3, 9, 10).

Oval cells are characterized by their unique morphology; ovoid nucleus, small size (relative to hepatocytes) and high nuclear to cytoplasmic ratio (13). Some studies showed that oval cells expressed CK8 and CK18, including other markers of oval cells such as OV-6, Thy-1, CK19, α-fetoprotein (AFP), c-kit, human epithelial antigen 125 and π-glutathione S-transferase (GST) (6, 14-17). In a normal adult liver, these cells are quiescent, existing in low numbers around the periportal region (14). Severe and chronic liver injury caused by drugs, viruses and toxins impairs hepatocyte proliferation and consequently leads to hepatic fibrosis. When the ability of hepatocytes to divide and replace damaged tissue is affected by these factors, the oval cells are induced to proliferate.

To date, there has been no report on the MBs and oval cells in carbon tetrachloride (CCl₄)-induced liver injury. Therefore, the induction and distribution of MBs and oval cells in CCl₄-induced rat liver fibrosis were investigated, and those MBs and oval cells expressing CK8 and CK18 were detected with immunohistochemistry and immunoblotting.

Materials and Methods

Experimental design. Male Wistar rats (n=64), weighing 200-220g, were housed in a room at 22±2°C with 12-hour light-dark cycle and were given food and water *ad libitum*. Animal experiments were performed in accordance with the NIH guidelines for the care

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Table I. *Grade of hepatic fibrosis in the CCl₄-induced rat hepatic injuries.*

Week	Grade of fibrosis[a]	Week	Grade of fibrosis
0	Grade 0	8	Grade 3
2	Grade 1-2	10	Grade 3
4	Grade 2	12	Grade 3-4
6	Grade 2-3	14	Grade 4

[a]Literature cited: (19) Grade 0, none; Grade 1, short collagenous septa extended from central veins; Grade 2, slender septa link the central veins, but lobular architecture is preserved; Grade 3, pseudolobuli are formed by thin septa; Grade 4, parenchyma is subdivided into smaller pseudolobuli by thin septa.

and use of laboratory animals. Fibrosis/cirrhosis was induced by intraperitoneal injection of 1.0 mg/kg body weight of 10% CCl₄ in olive oil 3 times a week for 14 weeks (18). Animals (8 at each time-point) were killed by cervical dislocation at 0, 2, 4, 6, 8, 10, 12 and 14 weeks, respectively.

Histology and immunohistochemistry. Liver pieces from each rat were rapidly removed at random and fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were cut to 4 μm in thickness. The sections were stained with hematoxylin and eosin (H&E). To quantify hepatic fibrosis, Grades 0-4 were used (19). For immunohistochemistry, sections of liver were deparaffinized in xylene, rehydrated in graded alcohol series, incubated in a solution of 3% hydrogen peroxide in methanol for 30 minutes and microwaved at 750 W for 10 minutes in 10 mmol/L citrate buffer, pH 6.0. Tissue sections were washed with PBS, and then immunostained with primary antibody. The primary antibodies used were monoclonal anti-Cytokeratin 8 and 18 at a dilution of 1:100, respectively (Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK). The antigen-antibody complex was visualized by an avidin-biotin-peroxidase complex (ABC) solution using an ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3, 3-diamino benzidine (Zymed Laboratories Inc., San Francisco, CA, USA). Tissue sections were then rinsed in distilled water and counterstained with Mayer's hematoxylin. For negative control, the primary antibody was replaced by phosphate-buffered saline.

Immunoblotting of CK18. CK18 protein was analyzed by modified immunoblotting described by Zatloukal *et al.* (8). In brief, a 5% (w/v) liver homogenate was prepared by sonication of frozen liver in sample buffer (10 mmol/L phosphate buffer containing 5% sodium dodecyl sulfate and 10% 2-mercaptoethanol). Proteins were precipitated by acetone, concentrations determined by the method of Braford and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis For immunoblotting, proteins were electro-transferred to PVDF membrane (Schleicher & Schuell, Dassel, Germany). After blocking, the membrane was incubated with the CK18 antibody (1:500) for 2 hours at room temperature. CK18 was detected using monoclonal mouse antibody against CK18 (Novocastra Laboratories Ltd). After washing in TBS, blots were incubated with Anti-Mouse IgG HRP Conjugated (Promega, Madison, WI, USA). Specific binding was detected

using the Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and exposure of the blots to Medical X-ray Film (Kodak, Tokyo, Japan).

Results

Histopathology. Hepatic fibrosis and cirrhosis were successfully induced by CCl₄ injection as described in our previous study (18), and shown in Table I. In this fibrosis/cirrhosis rat model, we found several cytoplasmic hyaline inclusions in hepatocytes at week 6 (Figure 1A). These were mainly detected in the perinuclear area of the hepatocyte near fibrous septa and were characterized by the well membrane-bound inclusion bodies. There was also a clear rim zone surrounding most of these bodies. These bodies were suspected as MBs. From week 6 to 14, most MBs were found in hepatocytes near fibrous septa, and the number and size of these increased in proportion to the development of fibrosis (Figure 1B). Hepatocytes sometimes contained both a hyaline body and a fatty droplet (Figure 1C).

Immunohistochemistry. By using monoclonal antibodies specific to CK8 and CK18, positive reactions for CK8 were in accordance with those of CK18 in the liver throughout the experiment. At week 0, there were very weak positive reactions for CK8 and CK18 in all hepatocytes. However, from week 4, positive reactions with irregular-shaped clumps of filaments were observed in the membrane and cytoplasm of damaged hepatocytes (Figure 1D). At week 6, well membrane-bound inclusions of different sizes were detected in addition to clumps of filament (Figure 1E). There was also an increased positive reaction of CK8 and CK18 in hepatocytes around fibrous septa (Figure 1F). From week 6 to 14, inclusion bodies and clumps of filaments had been

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Figure 1. *Mallory bodies and clumps of filaments are positive for CK8 and CK18 in CCl₄-induced liver fibrosis. (A) At week 6, several cytoplasmic hyaline inclusion bodies (small arrow) in hepatocytes were detected near the fibrous septa (FS). (B) As repeated hepatic injuries resulted in fibrosis, the number and size of inclusion bodies within a hepatocyte increased at week 14; the inset in the lower right corner of B shows the increased size of hyaline inclusion body. (C) Often, ballooning hepatocyte contains both hyaline body and fatty droplet. (D) Positive reactions for CK8 and CK18 with varying size and irregular clumps of filaments (open arrow) were detected at week 4. (E and F) MB (arrow head) positive for CK8 and CK18 are detected in addition to clumps of filament, which were mainly located near FS at week 6. (G) Hepatocytes showed diffusely strong positive reaction and contained inclusion bodies in the cytoplasm at weeks 12 and 14. (H) Hepatocytes had several inclusion bodies positive for CK8 and CK18 at weeks 12 and 14. (I) Ballooning hepatocyte with condensed nucleus strongly positive for CKs undergoing a sequence of necrosis. A-C, H&E staining; D-I, Immunohistochemistry. (Original magnification [A, B, D-F] x132; [C, G-I] x330)*
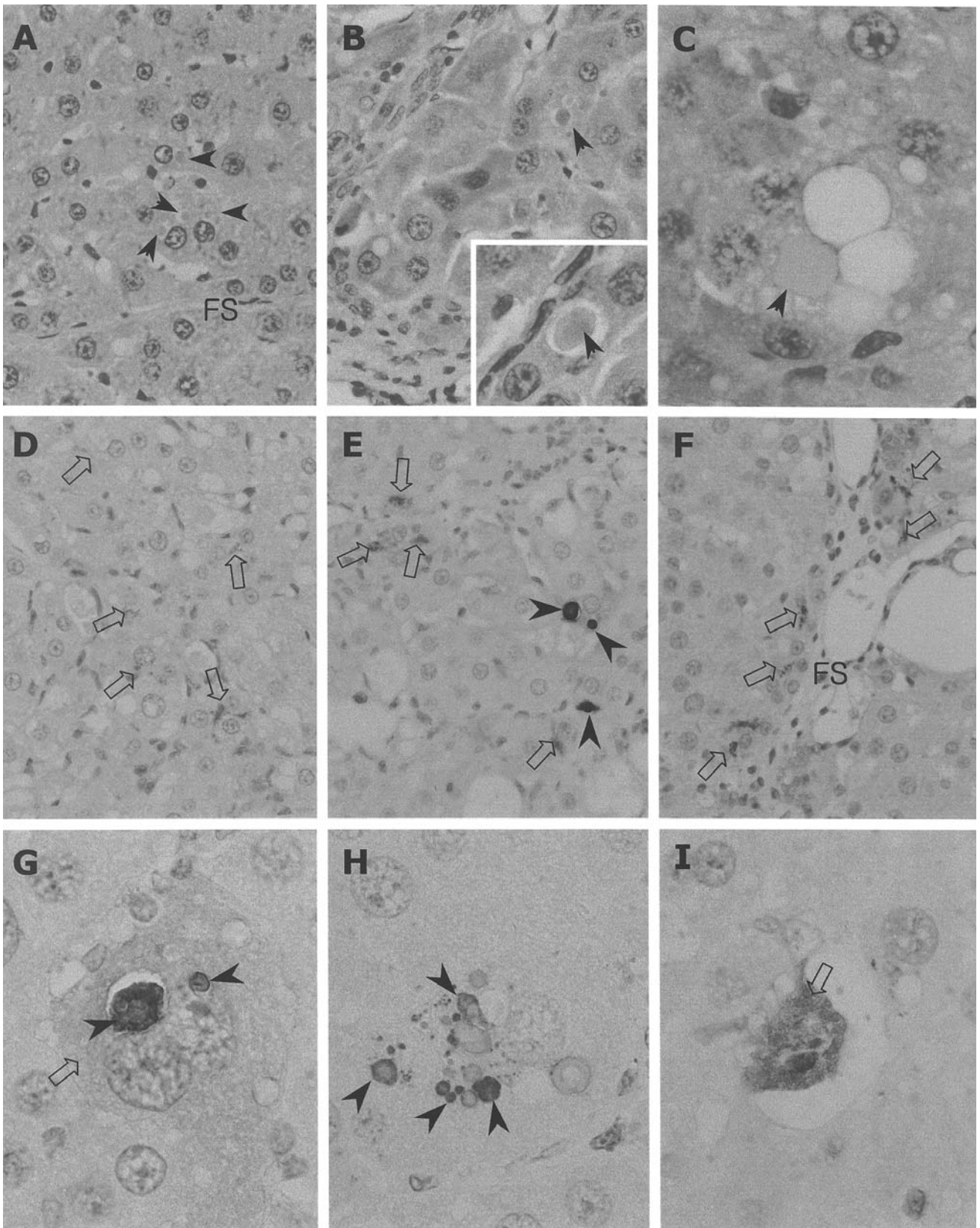
successively increased. Moreover, when cirrhosis occurred at weeks 12 and 14, inclusion bodies of various sizes, numbers and shapes were observed in the hepatocytes. Some hepatocytes showed a diffusely strong positive reaction and also contained inclusion bodies in the cytoplasm (Figure 1G). Other hepatocytes positive for CK8 and CK18 had several inclusion bodies (Figure 1H). Furthermore, hepatocytes with diffusely strong positive reaction underwent a sequence of degenerative changes (Figure 1I).

In addition to hepatocytes, another type of cell was positive for CK8 and CK18, and was detected around the central veins at week 2 (Figure 2A). Mitotic features were occasionally observed (Figure 2B) and were characterized by oval-shaped morphology with a prominent nucleolus and smaller size compared to the hepatocytes. They were diffusely strongly positive for CK8 and CK18 and were located mainly near the damaged area (Figure 2C). At week 6, there was an increase of these cells around the central veins and they were detected along or in the fibrous septa from weeks 8 to 10 (Figure 2D-2F). When cirrhosis occurred at week 12, most of these cells were located in the fibrous septa (Figure 2G). Moreover, these cells were detected around newly-formed bile ductules and increased in number (Figure 2H). However, epithelial cells forming bile ductules were not positive for CK8 or CK18. Only those oval-shaped cells not constituting bile ductules were positive in the fibrous septa (Figure 2I). From the above observations, it can be concluded that the hyaline bodies in the cytoplasm were MBs, and that oval cells and MBs expressed CK8 and CK18 simultaneously.

Immunoblot. Using the monoclonal antibody of CK18, specific bands were detected in the liver homogenates at weeks 0, 4, 8, 12 and 14 (Figure 3). A very light single band of CK18 was detected in the homogenate of control livers (week 0). As hepatic injuries developed to fibrosis and cirrhosis, a much more prominent single band of CK18 was detected.

Discussion

MBs represent cytoplasmic hyaline inclusions in hepatocytes characteristically observed in alcoholic liver disease (1-3, 8, 9, 12). Many studies have been performed to clarify the formation, composition and pathogenesis of MBs, but none of these studies has accounted for the whole phenomenon (1, 9, 20). Recently, it has become accepted that MB is a prominent feature of alcoholic liver disease as well as of non-alcoholic liver disease (1, 2, 9). Furthermore, MBs have been observed in other cell types such as muscular fibers, parathyroid chief cells, alveolar pneumocytes and lung carcinoma cells (2, 21). Experimentally, MBs have been induced by chronic intoxication with griseofulvin or DDC (3,5-diethocycarbonyl-1,4-dihydrocollidine), thus providing

an experimental system for study of MB pathogenesis in many studies (9, 22, 23). However, there have been no reports on the induction of MB using a CCl_4 rat model. The rat model used in the present study also allows many investigators to simultaneously perform several experiments on the hepatic fibrosis and pathogenesis of MB, including oval cells.

Several studies have demonstrated that CK8 and CK18 are involved in MB formation (1, 3, 4, 6, 8-11, 24). Yuan and colleagues have reported that MB formation was related to the cytoskeletal changes involving intermediate filaments in hepatocytes, and that injured hepatocytes eventually showed MB formation (10). Morphologically, MBs can be classified as type I (bundles of filaments in parallel arrays), type II (randomly oriented filaments) and type III (granular and amorphous material) and are usually present in enlarged, ballooned hepatocytes, most of which look vital and have large nuclei and nucleoli (1). In our study, there were not only type II and type III MBs, but also dense CK8- and CK18-positive vesicles in the liver, not previously described.

Recently, one study has reported that increased CK8 and CK18 protein levels and formation of MBs in primary biliary cirrhosis were mainly observed in the acinar zone 1 or in the periphery of cirrhotic nodule, and were considered to be due to cholestasis and associated with the highest intrahepatic bile acid levels (24). In our study, as hepatic fibrosis developed progressively, MB formation was increased and was primarily observed in hepatocytes around necrotic and fibrotic areas. According to immunoblotting results, CK18 levels also increased. Although MBs are found in severely damaged hepatocytes, these cells are not necessarily prone to death, since hepatocyte-containing MBs are viable and MB formation may cease and revert after the disappearance of the damaging agent (25). Similarly, our study indicates that the increase of CK8 and CK18 expression in hepatocytes appears to be related to MB formation, but is not associated with hepatic necrosis. However, most enlarged hepatocytes with abundantly

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Figure 2. *Oval cells positive for CK8 and CK18 in CCl_4-induced hepatic injuries. Most oval cells (arrow) showed strong positive reaction. (A) An oval cell was detected around central veins (CV) at week 2; the inset in the lower left corner of A shows an oval cell with a prominent nucleolus. (B) Dividing oval cells were occasionally observed at week 4. (C) Oval cells with strong positive reaction were mainly located in the CCl_4-damaged area at week 6. (D-F) Increase of oval cells was detected along or within the fibrous septa from weeks 8 to 10. (G-I) Most oval cells were located in the fibrous septa and near or around newly formed bile ductules (*) at weeks 12 and 14. Immunohistochemistry. (Original magnification [A, C, E, I] x132; [B, inset in A] x330; [D, G, H] x66; [F] x33).*
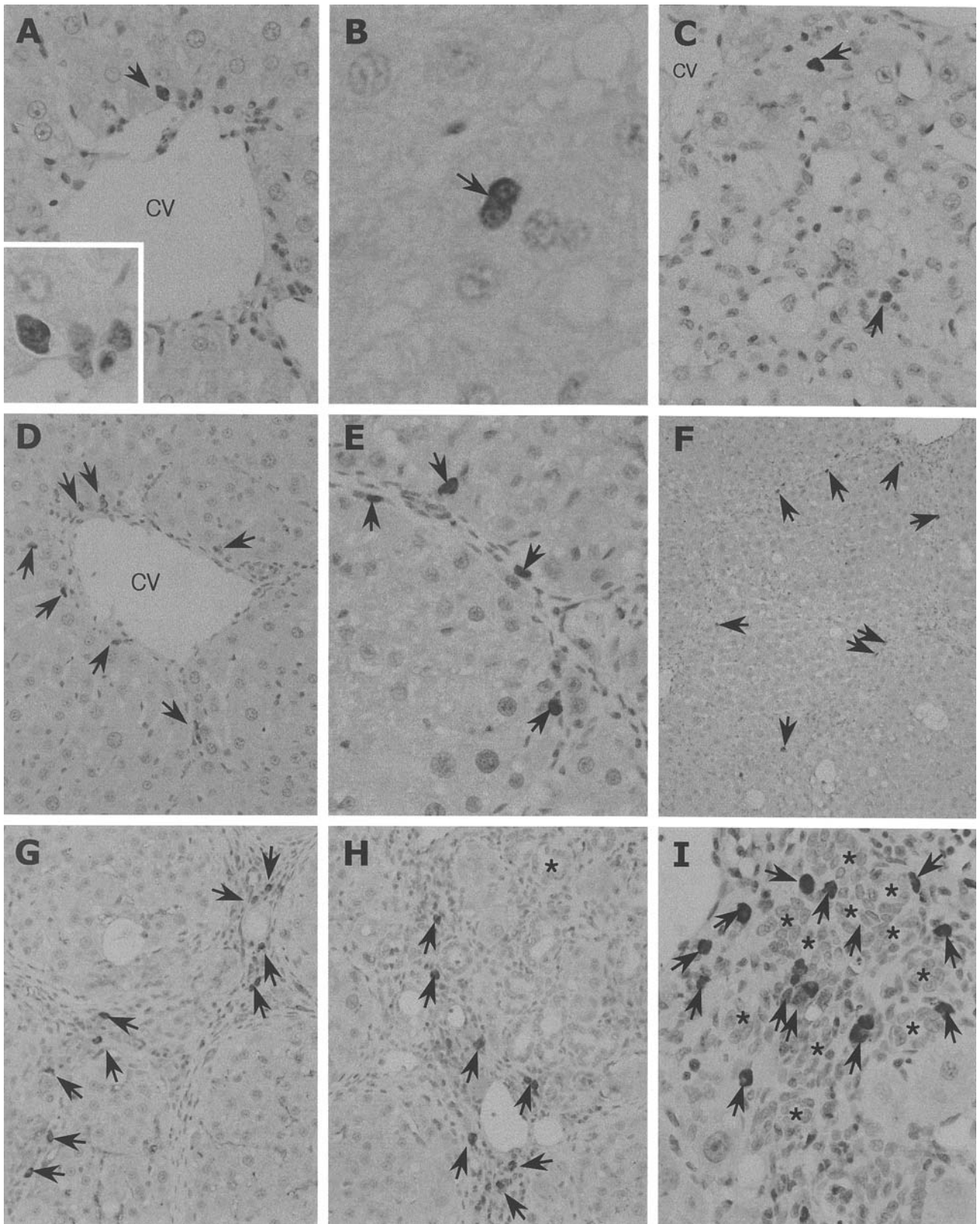
Figure 3. *Immunoblot analysis of rat liver homogenates with CK18 antibody. A single major band increased in intensity with duration of CCl₄ intoxication (0, 4, 8, 12 and 14 weeks).*

expressed CK8 and CK18 did undergo necrosis. Therefore, it was considered that a specific type of liver cell injury increased CK8 and CK18 levels in enlarged hepatocytes, with or without MBs, consequently killing the affected hepatocyte. The present study also provides new insights into the involvement of CKs in the cellular response to toxic injury. We found that hepatocytes respond to a toxic challenge, in this case of CCl₄, with up-regulation of CK8 and CK18 synthesis, in agreement with other similar experiments (9, 10, 24).

Oval cells in rodent liver are small, undifferentiated epithelial cells with an ovoid nucleus and scanty cytoplasm with a large nucleus-to-cytoplasm ratio (26, 27). Numerous studies have reported that oval cells are hepatic progenitor cells that can differentiate to the hepatocyte and the biliary lineage (27, 28). The origin of oval cells is known, and they can be of endogenous (Hering canal cell) or exogenous origin (most probably bone marrow) (14, 27, 29). Regardless of the hepatic oval cell origin, it is well established that oval cell proliferation occurs when the replicative capacity and function of hepatocyte are impaired. Oval cells are resistant to the effects of hepatotoxins/carcinogens, thus, they proliferate and migrate throughout damaged liver lobules to replace lost parenchyma (14). Oval cells are known to have several different combinations of the phenotypic markers: AFP, Thy-1, CK8, CK18, CK19, OV-6 and π-GST (6, 14-17). In the portal lesions of human patients and hamsters with chronic liver disease, 3 oval cell types (primitive, hepatocyte-like and ductular-like type) have been identified and distinguished by ultrastructural analysis and immunohistochemistry (30, 31). These analyses have indicated that primitive-type cells not expressing AFP, CK19, OV-6 or π-GST were located in close proximity to proliferating bile ducts and in acinar arrangements around hepatocytes. Hepatocyte-like cells expressing only AFP were located in areas of ductular proliferation, next to cells comprising small ductules, and ductular-like cells, negative for AFP but expressing CK19, OV-6 and π-GST, were present in ducts. However, in our study, oval cells expressing CK8 and CK18 were present in the injured hepatocyte areas and in close proximity to proliferating bile ducts on the portal triad and in and around fibrotic septa. Therefore,

distinguishing oval cell types as described above was controversial in our case. However, the features shown in Figure 2 led to the hypothesis that oval cells which are CK8- and CK18-positive, but which do not constitute bile ductules, are hepatocyte progenitor cells and ductule epithelial cells. Moreover, since normal hepatocytes have CK8 and CK18 in their cytoplasm, CK8 and CK18 should be expressed in oval cells if oval cells are involved in hepatocyte regeneration.

Several studies have stated that oval cells were located in close association with inflammatory cells (32, 33). Evarts *et al.* have shown that hepatic stellate cell (HSC) proliferation is closely associated with oval cell proliferation (34). Nagy *et al.* (35) have suggested that HSC-derived hepatocyte growth factor may cause oval cell proliferation *via* the paracrine activation of c-met. Fausto *et al.* found that massive necrosis by CCl₄ elicited an inflammatory reaction, but there was no evidence that liver progenitor cells were activated to generate replicating hepatocytes during the growth process (36). In our study, oval cells were mainly located in the fibrotic area in which HSC proliferation might have occurred. This supports former theories that oval cells may be also related to hepatic stellate cells and fibrosis.

In conclusion, we report the induction and distribution of MBs and oval cells in CCl₄-induced rat liver fibrosis. This represents the first CCl₄ experimental *in vivo* model of MB induction, which will be useful for further investigations on the model of formation and progression of MB and its role in the pathogenesis of cell damage. Furthermore, this model provides an opportunity to study fibrosis and oval cells simultaneously.

Acknowledgements

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