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Letter to the Editor

In situ monitoring of caspase activation in hepatobiliary diseases

Dear Editor,

Apoptosis has been implicated in the pathogenesis of many diseases including various forms of liver failure. The apoptotic process is essentially regulated by cysteine proteases, called caspases, which cleave several intracellular proteins leading to the final demise of the cell. Despite the rapid elucidation of apoptotic signaling cascades, however, almost no information exists about the activation of caspases *in situ*.[1] In this study we have investigated the occurrence of caspase activation in hepatobiliary diseases using a monoclonal antibody directed against a caspase-generated neoepitope of cytokeratin-18 (anti-frag-CK18).

CK18 is a type I intermediate filament, which is expressed in the liver, exocrine pancreas, intestine and other epithelial tissues. Although the full scope of its physiological role is unknown, one clear function of CK18 is to help maintain epithelial cell integrity. During apoptosis CK18 aggregates and becomes degraded, an event which may contribute to the morphological alterations of apoptosis. Caspases cleave CK18 at Asp398 generating a 45 kDa intermediate fragment, which is further cut at Asp237 into two fragments of approximately 26 and 21 kDa (Figure 1A).[2,3] This cleavage pattern is highly specific to apoptosis and also obtained *in vitro* using recombinant caspase-3 and caspase-6. In Western blot analyses we found that the 21 kDa and the intermediate fragment, but not full-length CK18 were selectively recognized by anti-frag-CK18 (data not shown), indicating that the antibody binds to a neoepitope at the Asp398 cleavage site. By immunocytochemistry, we showed that HepG2 hepatoma cells induced to undergo apoptosis by various proapoptotic stimuli including anti-CD95 revealed a strong reactivity with anti-frag-CK18 (Figure 1B). In contrast, normal viable cells or cells induced to undergo necrosis by hydrogen peroxide were not stained, demonstrating that the antibody is useful for the detection of apoptotic cells.

In several either autoimmune, viral or drug-induced liver diseases increased apoptosis is likely to play an important pathogenic role. For instance, enhanced apoptosis has been suggested to be responsible for bile duct injury and loss of cholangiocytes in primary biliary cirrhosis or liver cell damage in Wilson's disease, hemochromatosis, viral hepatitis and acute hepatic failure.[4] However, it is virtually unknown, whether and to which extent caspases are activated in these pathologies. We therefore investigated the ability of the antibody to detect caspase activation in formalin-fixed liver biopsies. With an anti-pan-CK18 antibody it was confirmed that CK18 is expressed by human hepatocytes (Figure 1C,a). Using anti-frag-CK18 there was no immunoreactivity evident in normal liver tissue (Figure 1C,b). In contrast, in several diseases, such as primary sclerosing cholangitis, hepatitis B and -C virus infection, drug-induced hepatitis and alcoholic liver disease, apoptotic cells were detected in liver sections. In liver biopsies of HBV or HCV infection staining was found only in a few scattered hepatocytes (data not shown). A more intense immunoreaction was apparent in tissue biopsies of alcoholic liver diseases (Figure 1C,c). The antibody appeared to stain also Mallory bodies, which contain an abnormal aggregation of keratin complexes particularly in alcoholic liver disease. In advanced alcoholic liver disease fat-transformed hepatocytes stained positive (Figure 1C,d), and in some cases the majority of the hepatocytes exhibited a diffuse cytoplasmic staining. Although we did not determine the number of positive cells, our results show that CK18 cleavage is indeed increased in liver disease. It should be kept in mind that the approach of quantifying apoptosis certainly underestimates the rate of apoptosis in the liver, because apoptotic bodies are rapidly taken up by phagocytes.

Our data demonstrate that the anti frag-CK18 antibody can be employed to monitor caspase activation and to detect an early phase of apoptosis in cultured cells as well as formalin-fixed tissue biopsies. Currently, the most widely used method for visualizing apoptotic cells is the TUNEL technique which has several known pitfalls.[5,6] The technique is based on detection of DNA fragmentation which is a late event in apoptosis and occurs also during necrosis or autolysis. It has been also found that some forms of apoptotic cell death, even in hepatocytes, are not associated with DNA fragmentation. Comparing the TUNEL method and immunostaining with anti-frag-CK18, we found that a much larger number of cells were stained by the endlabeling technique. We speculate that the artificially high TUNEL reaction might be due to labeling conditions, autolysis and the release of endogenous DNases. Because apoptosis offers new possibilities for therapeutic interventions, it is crucial to discriminate it from other types of cell death. Therefore the use of cleavage site-directed antibodies against caspase substrates may be a more reliable tool to study apoptosis *in situ*. The detection of caspase-mediated CK18 cleavage may be of particular interest from a clinical perspective, because proteolytic CK18 fragments are found in sera of patients with liver diseases and a variety of carcinomas.

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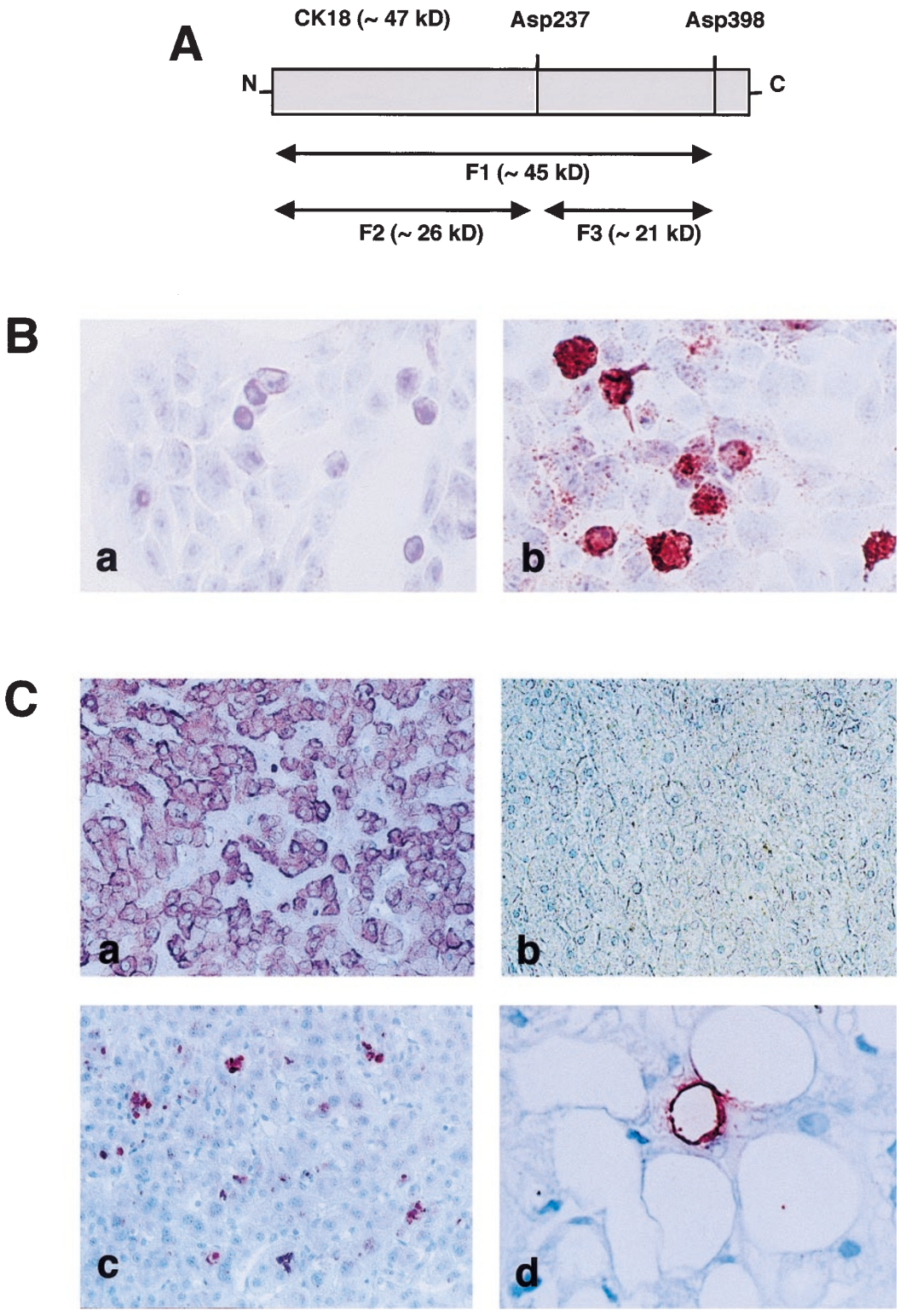

Figure 1 (**A**) Schematic presentation of the CK18 structure and its caspase cleavage sites. CK18 is cleaved into three fragments (F1–F3). The anti-frag-CK18 antibody detects a neoepitope at the Asp398 cleavage site. The antibody was originally generated by immunizing mice with a cytokeratin preparation from colon carcinomas cells[7] and is now available from Roche Molecular Biochemicals. (**B**) Immunostaining of HepG2 hepatoma cells. Normal viable cells were not stained by the antibody (a). In contrast, apoptotic cultures treated with anti-CD95 revealed a strong immunoreactivity (b). (**C**) Detection of caspase activation with anti-frag-CK18 in liver tissues. Biopsies collected from patients (n=56) with different liver diseases were investigated. Following rehydration of the paraffin sections, antigen retrieval and incubation with the primary antibody, CK18 cleavage was detected by the avidin-biotin based immunoperoxidase technique (red staining). Detection of full-length CK18 expression in the liver by an anti-pan-CK18 antibody (a). With anti-frag-CK18 no immunoreactivity was found in healthy liver (b). Detection of caspase activity in alcohol-induced hepatitis (c) and advanced alcoholic liver disease (d)