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Caspase-Cleaved Cytokeratin 18 and 20 S Proteasome in Liver Degeneration

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Apoptosis of epithelial hepatocytes plays a pivotal role in acute as well as in chronic liver diseases. The cleavage of cytokeratin-18 (CK-18) by caspases is an early event in the apoptotic process. We therefore sought to investigate serum levels of CK-18 and 20S proteasome in patients with liver cirrhosis, primary graft dysfunction (PDF), and acute liver failure (ALF), and in healthy volunteers. Enzyme-linked immunosorbent assay (ELISA) was utilized to measure the concentration of M30, a fragment of CK-18 cleaved at Asp396 (M30 neoantigen), and the concentration of 20S proteasome. Serum levels of the CK-18 neoepitope M30 were significantly increased in ALF, primary graft dysfunction, and liver cirrhosis vs. healthy controls (1,993.6 $\pm$ 124.7 U/L, 2,238.1 $\pm$ 235.9 U/L, and 673.6 $\pm$ 86.5 U/L vs. 66.8 $\pm$ 29.1 U/L, respectively, $P < 0.001$). Similar results were detected with the evaluation of 20S proteasome (124,014.5 $\pm$ 13,235.6 ng/mL, 76,993.2 $\pm$ 15,720.1 ng/mL, and 2,395.9 $\pm$ 1,098.2 ng/mL vs. 1,074.5 $\pm$ 259.4 ng/mL, respectively; $P < 0.001$). Detection of CK-18 neoepitope M30 and 20S proteasome may represent a novel marker of tracing apoptotic epithelium, respectively mirroring degenerative liver processes in affected patient population. J. Clin. Lab. Anal. 21:277–281, 2007. © 2007 Wiley-Liss, Inc.

Key words: epithelial cell; degeneration; apoptosis; proteasome; liver; caspase; cytokeratin

INTRODUCTION

Apoptosis has been described in acute and chronic liver injury (1). Acute liver failure (ALF) is characterized by a rapidly progressing, life-threatening, but potentially reversible, deterioration of liver function. Tissue injury, ischemia-reperfusion injury, viral or bacterial infections, and toxins are known agents to cause this life-threatening condition (2,3). Autopsy studies showed that apoptosis is deemed to be a major mechanism of liver dysfunction in ALF (4). These findings were verified immunohistologically by detection of cytokeratin-18 (CK-18) (5,6).

Although primary graft dysfunction (PDF) occurs in up to 30% of all allografts, the mechanisms leading to this are still unclear (7). However, there is evidence that apoptosis is involved in the pathogenesis of PDF (8).

In liver cirrhosis, the role of apoptosis is well characterized. However, serum levels of CK-18 and 20S proteasome have not been described in this disease entity before. Apoptosis induced by either death-inducing receptors or other stimuli leads to activation of specific caspases. Subsequently, these cells are eliminated by an intrinsic suicide program, resulting in deoxyribonucleic acid (DNA) fragmentation, nuclear condensation, cytoskeletal reorganization, plasma

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membrane blebbing, and loss of cell adhesion (9). CK-18 is a type I intermediate filament protein and the major component of single-layer and glandular epithelial cells. During apoptosis after initiation of epithelial effector caspases 3, 6, and 7, CK-18 is cleaved into proteolytic fragments, which diffuse into the serum. Recently, a monoclonal antibody that selectively recognizes a neoepitope of CK-18 after caspase-induced cleavage during apoptosis, has become available (10,11).

The proteasome pathway is deemed pivotal in the degradation of epithelium and general wasting syndrome, a condition present in patients with ALF (12,13). Proteasome is a multicatalytic proteinase complex, localized in the cytosol as well as in the nucleus of all eukaryotic cells, and is crucially involved in the enzymatic degradation of ubiquitinated proteins. Moreover, it is a controller of numerous physiological processes, including signal transduction, transcriptional activation, cell-cycle progression, and certain immune cell functions. The proteasome is made up of a cylindrical-shaped core particle, the so called 20S proteasome, which consists of two sets of seven different a and b subunits assembled in four heptameric rings. Addition of 19S regulatory proteins to the end of the 20S proteasome yields a 26S complex. The 19S subunit contains six adenosine triphosphatases (ATPases) and is essential in the ubiquitin-proteasome proteolysis pathway, whereas the 11S proteasome (PA28, REG), which is inducible by interferon-γ, has been implicated in antigen processing and formation of immunoproteasome (14,15). Increased levels of circulating 20S proteasomes were recently proposed as markers of cell damage and immunological activity in various autoimmune diseases and sepsis (16,17). The aim of this study was to determine if apoptosis-specific derivative serum content of CK-18 neoepitope M30 and 20S proteasomes in patients with liver cirrhosis, acute graft dysfunction, or ALF is increased as compared to healthy controls.

MATERIALS AND METHODS

Patients

The Institutional Review Board of the General Hospital, University of Vienna Medical School, Vienna, Austria, approved the study protocol. Procedures were performed in accordance with the Helsinki Declaration (as revised in Edinburgh 2000). All study and control subjects or their legal designees signed an informed consent. Both ALF and PDF were defined by meeting three of the following four diagnostic criteria: plasma disappearance rate of indocyanine green lower than 10%/min; total serum bilirubin higher than 10 mg/dL; aminotransferase levels higher than 1,500 U/L and international normalized ratio of prothrombin (INR) higher than 1.5. Investigations were carried out with sera from 12 patients each with ALF, PDF, liver cirrhosis, and 12 healthy controls. The demographic and clinical features are shown in Table 1. Blood sampling was performed meeting the exclusion criteria of freedom of radiation and antineoplastic chemotherapy or detection of impaired hepatic macrocirculation by Doppler ultrasound. Blood samples from ALF patients were gained within 24 hr after admission to the transplant intensive care unit (ICU), from PDF patients within 4 days after orthotopic liver transplantation (OLT), and from liver cirrhosis patients during an ambulatory visit.

TABLE 1. Profiles of patients with liver cirrhosis, PDF, and ALF*

	Liver cirrhosis patients (n = 12)	PDF patients (n = 12)	ALF patients (n = 12)
Age (years, median)	46	50.5	33
Sex (%male)	70	80	70
Days in ICU (median)	0	10	9
Survival (90 days, %)	100	75	75
Liver regeneration (%)	0	75	40
Need for OLT (%)	100	0	50
Origin of liver failure			
Alcoholic cirrhosis	12		
Primary graft dysfunction (PDF)		12	
Acute hepatitis A			1
Acute hepatitis B			3
Autoimmune hepatitis			2
Idiosyncratic hepatitis			1
Amanita poisoning			4
Paracetamol poisoning			1

*The control group contains 12 apparently healthy donors.

Quantification of CK-18 M30 Neoepitope

Serum samples were aliquotted and kept frozen. Circulating soluble CK-18 M30 neoepitope was measured by a commercial enzyme-linked immunosorbent assay (ELISA) purchased from Peviva AB (Bromma, Sweden). This ELISA uses a monoclonal antibody recognizing an epitope on the 238–396 fragment of CK-18 as catcher and horseradish peroxidase-conjugated M30 as detector. M30 antigen levels are expressed as Units per liter (1 Unit (U) corresponds to 1.24 pmol of a synthesized peptide of the M30 recognition motif according to the manufacturer). The sensitivity of the ELISA is 30 U/L. The intra- and interassay coefficients of variation of the ELISA were 0.7–5.8% and 2.8–4.8%, respectively. The amount of protein in each sample was calculated according to a standard curve of optical

density values constructed for known levels of CK-18 neoepitope M30.

Determination of Circulating 20S Proteasome

Microtitration plates were coated overnight with the mouse monoclonal antibody to 20S proteasome subunit a6 (HC2) purchased from Affinity Research Products Ltd (Exeter, UK) 1:4,500 in carbonate buffer, pH 9.6. Remaining binding sites were blocked with 0.5% fetal calf serum (FCS) in phosphate buffered saline (PBS), pH 7.4. Human sera samples were diluted 1:20 and applied to each well for 3 hr at room temperature. Standard curves were established using 20S proteasome in a concentration of 5,000 ng/mL to 78 ng/mL (six linear dilution steps). After a washing step, a rabbit polyclonal antibody to 20S proteasome a/b subunits (Affinity Research Products Ltd) was added for an incubation period of 2 hr at room temperature. Following another washing step peroxidase-conjugated mouse anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA) was used for the detection of the antigen for an incubation period of 1 hr at room temperature. The bound antibodies were detected with tetramethylbenzidine as substrate. The reaction was stopped with sulfuric acid and optical density (OD)-values were determined at 450 nm. To exclude the possibility of nonspecific binding, we tested bovine serum albumin as control protein instead of 20S proteasome and did not observe any positive reaction (data not shown).

Statistical Analysis

Results are presented as median and range. Due to the relatively small sample size and nonparametric distribution of the data, Mann-Whitney-U-test was used to calculate significance, and a P value of 0.01 was considered to be statistically significant.

RESULTS

Increased Concentration of CK-18 in Liver Failure

Figure 1 demonstrates a significant increase in the concentration of CK-18 M30 neoepitope in sera from patients with liver cirrhosis PDF and ALF as compared to healthy controls. The mean serum levels were as follows: for ALF, $1{,}993.6 \pm 124.7$ U/L; for progressive graft dysfunction, $2{,}238.1 \pm 235.9$ U/L; for liver cirrhosis, 673.6 ± 86.5 U/L; and for controls 66.8 ± 29.1 U/L.

Increased Levels of 20S Proteasome in Liver Failure

The results depicted in Fig. 2 demonstrate a significant increase in the concentration of circulating 20S proteasomes in the sera from patients with ALF, PDF, liver cirrhosis vs. controls ($124{,}014.5 \pm 13{,}235.6$ ng/mL, $76{,}993.2 \pm 15{,}720.1$ ng/mL, $2{,}395.9 \pm 1{,}098.2$ ng/mL vs. $1{,}074.5 \pm 259.4$ ng/mL).

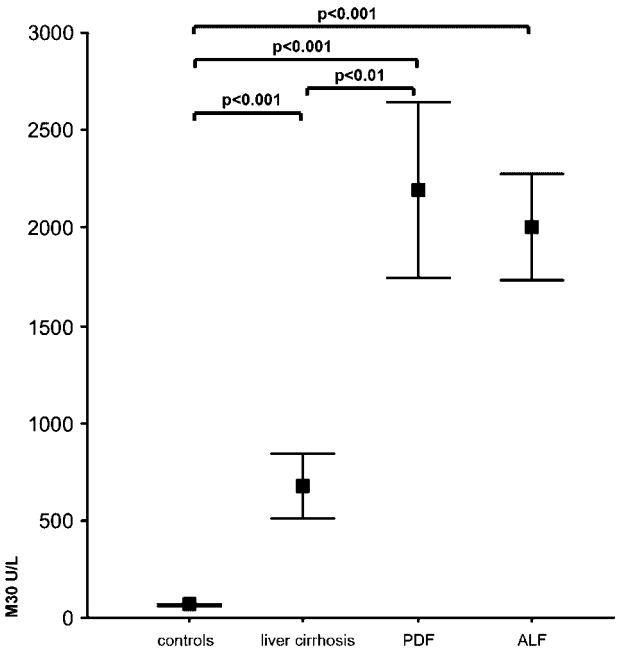

Fig. 1. Concentration of circulating cytokeratin 18(CK-18) in the sera of healthy controls (n = 12), liver cirrhosis (n = 12), primary graft dysfunction (n = 12), and acute liver dysfunction (n = 12). Data are given in mean ± SEM.

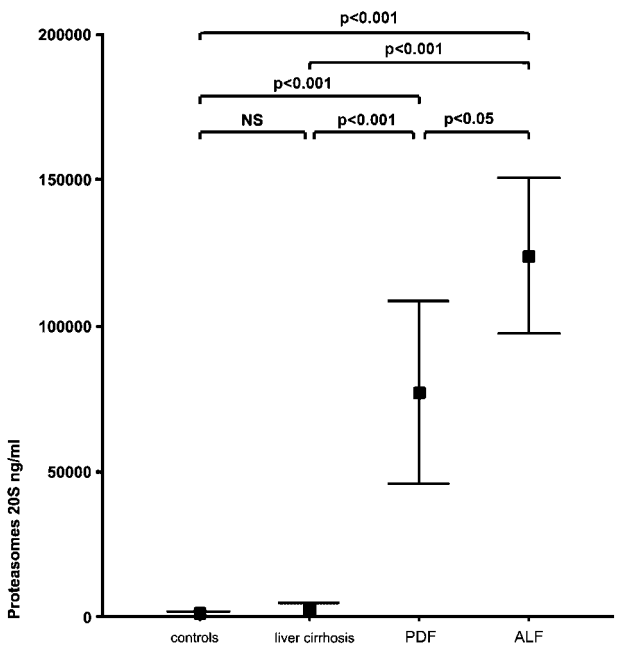

Fig. 2. Concentration of circulating 20S proteasome in sera of healthy controls (n = 12), liver cirrhosis (n = 12), primary graft dysfunction (n = 12), and acute liver dysfunction (n = 12). Data are given in mean ± SEM.

DISCUSSION

Apoptosis has been implicated in the pathogenesis of many diseases including various forms of liver failure. The apoptotic process is essentially regulated by intracellular proteases, called caspases, which cleave several vital proteins. Despite the rapid elucidation of apoptotic signaling cascades, however, almost no information exists about the involvement of apoptosis specific CK-18 and proteasome activation.

In the present study, a monoclonal antibody was employed that selectively recognized cleavage site–specific fragments of the caspase substrate CK-18. This M30 antibody was evaluated as feasible for use in detecting cells of eukaryotic origin undergoing apoptosis (18). Our data reveal for the first time that hepatobiliary diseases are associated with elevated serum CK-18. CK filaments have been observed to aggregate rapidly in apoptotic cells, due to hyperphosphorylation. CK-18 is cleaved by caspases, liberating a fragment of approximately 20 kD containing the M30 neoepitope, which is specifically recognized by the M30 monoclonal antibody. In the end phase of liver degeneration, e.g., necrosis, the M30 binding is lost. The capacity of the M30 antibody to identify apoptotic cells has been verified in several diseases and the M30 ELISA is suggested prior to this observation as a high-throughput assay for screening of proapoptotic drugs. CK-18 has a widespread distribution and is found in practically all simple, nonstratified, ductal and pseudostratified epithelia of the gastrointestinal tract, liver, female genital tract, urinary tract, and respiratory tract. Therefore, the definite organ source of the neoepitope is not identified in our study. Likely, it is derived from hepatocytes, because our patients clinically presented liver failure and the serum levels were 10-fold higher than in septic patients (19). Moreover, a recent work by Ramskogler et al. (20) found highly significant positive correlations between CK-18 serum levels and liver enzymes indicative for degenerative liver diseases.

Concurrent with our CK-18 data, increased levels of soluble serum 20S proteasome were found in patients with ALF. Notable was that soluble 20S proteasome evidenced a 100-fold increase in ALF as compared to healthy humans and a 4-fold increase was determined in septic patients (16). Using a self-constructed ELISA system, we measured concentrations of 120–140,000 ng/mL of 20S immune proteasome in liver cirrhosis, primary graft dysfunction, and ALF. In general, the results of a sandwich ELISA depend on the quality of the capture and detection antibodies and the quality of antigen used for the standard curve. Therefore, it is not surprising that different proteasome concentrations were measured for healthy controls by other assays. In particular, differences could be attributed to the detection of different amounts of the intact protease complex or degradation products, such as rings or single subunits of the proteasome. However, the differences in the concentrations of 20S proteasome in our study (in the control group) are comparable to the reference range of Stoebner et al. (21) and Dutaud et al. (22). Like autoimmune disorders, sepsis, trauma, and neurodegenerative diseases are characterized by wasting syndrome and activation of white blood cells. If we interpret our data correctly we hypothesize that general degeneration of epithelial cells, endothelial cells, and hemolysis of red blood cells leads to spillage of proteasomes into the vascular bed (23). Increased values of 20S proteasome are therefore self-evident. The role of 20S proteasome was shown to correlate with disease activity in autoimmune diseases. To estimate the contribution of cellular damage to heightened 20S proteasome levels, we determined their concentration in patients who suffer from liver cirrhosis caused by chronic alcohol abuse. A slight increase in 20S proteasome levels in these patients was determined. This led us to the assumption that cellular damage rather than immunological activity is causative of the clearly increased 20S proteasome levels in acute liver failure and primary graft dysfunction.

However, muscle proteolysis, another clinical feature, is often found in catabolic conditions and should be taken into consideration for elevated 20S proteasome content because the ubiquitin-proteasome pathway plays a pivotal role in the degradation of myofibrillar proteins during liver failure, sepsis syndrome, and kidney disease (24–26).

In conclusion, we evidenced increased serum levels of CK-18 and 20S proteasomes in patients suffering from liver degeneration. We suggest that these new markers provide a simple and efficient tool in evaluating degenerative processes in these disease entities. Our study is, however, limited through the unknown variables of circadian rhythm and half-life of the CK-18 and 20S proteasome, as the clearance mechanism of these substances is still unknown. Furthermore, the possible effect of myofibrillar proteolysis on 20S proteasome serum levels as well as a possible correlation with other inflammation markers like C-reactive protein or white blood count should be addressed in further studies. Our results, however, warrant further clinical studies to assess the role of 20S proteasome and CK-18 in liver degeneration and their possible role in monitoring.

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