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Individuals with Primary Sclerosing Cholangitis have Elevated Levels of Biomarkers for Apoptosis but Not Necrosis

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Abstract

Background and Aim—Hepatocyte apoptosis or necrosis from accumulation of bile salts may play an important role in the disease progression of primary sclerosing cholangitis (PSC). The aim of the current study was to measure serum markers of hepatocyte apoptosis (cytokeratin 18 fragments - K18) and necrosis (high-mobility group protein B1 - HMGB1) in adults with PSC and examine the relationship with disease severity.

Methods—We measured serum levels of K18 and HMGB1 in well phenotyped PSC (N=37) and 39 control subjects (N=39). Severity of PSC was assessed biochemically, histologically and PSC Mayo Risk Score. Quantification of hepatocyte apoptosis was performed using *TUNEL* assay.

Results—The mean age of the study cohort was 49.7 ± 13.3 years and comprised of 67% men and 93% Caucasian. Serum K18 levels were significantly higher in the PSC patients compared to control (217.4 ± 78.1 vs. 157.0 ± 58.2 U/L, p-val=0.001). However, HMGB1 levels were not different between the two groups (5.38 ± 2.99 vs. 6.28 ± 2.85 ng/mL, p-val=0.15). Within the PSC group, K18 levels significantly correlated with AST (r=0.5, p-val=0.002), alkaline phosphatase (r=0.5, p-val=0.001), total bilirubin (r=0.61, p-val= <0.001), and albumin (r=−0.4, p-val =0.02). Serum K18 levels also correlated with the level of apoptosis present on the liver biopsy (r=0.8, p-val = <0.001) and Mayo Risk score (r=0.4, p-val=0.015).

Conclusion—Serum K18 but not HMGB1 levels were increased in PSC and associated with severity of underlying liver disease and the degree of hepatocyte apoptosis.

Keywords

Serum K18; TUNEL; HMGB1; apoptosis; hepatocyte necrosis; ulcerative colitis

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic liver disease characterized by fibrosis of intra- and/or extra- hepatic biliary tree resulting in the development of diffuse biliary strictures [1,2]. Biliary structuring leads to intrahepatic cholestasis that can lead to cholangiocyte and hepatocyte cell death. The precise pathophysiologic mechanisms through which this occurs, however, remains unclear. Liver has a large population of immune cells including Kupffer cells, natural killer (NK) cells, and natural killer-T (NKT) cells that produce signaling molecules such as tumor necrosis factor alpha (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand [3–5]. These molecules often perpetuate the initial injury from intrahepatic cholestasis through apoptotic pathways from accumulation of endogenous toxic bile acids [6]. One study that examined explanted liver tissue of primary biliary cirrhosis (PBC) and PSC patients showed that apoptosis in the bile ducts was significantly increased in PBC and not PSC patients [7]. Therefore, it has been suggested that apoptosis did not play a central role in PSC [8]. However, a recent study measured several serum markers of apoptosis (soluble Fas, M30 and M65) in patients with immune-mediated liver diseases and reported that patients with PSC had increased serum markers of apoptosis that correlated with disease activity [9].

Biochemically, apoptosis is commonly initiated and executed by activation of intracellular enzymes termed caspases [10]. Hepatocytes contain in their cytoplasm, a cytoskeletal system consisting of intermediate filament proteins primarily made up of keratins, K8 (previously called CK8) and K18 (previously called CK18) which play an important functional role in the integrity and mechanical stability [10–12]. Induction of apoptosis in liver disease results in early cleavage of K18 by caspases [10]. These fragments are stable to proteolysis and are released into the circulation after hepatocyte plasma membrane disintegration during later stages of apoptosis [10]. The serum K18 fragment levels can be measured by a commercially available ELISA assay, and has shown to be altered in some liver disorders associated with hepatic inflammation and injury [9,13,14].

Hepatocyte death can also occur through necrosis, but the role of hepatocyte necrosis in liver disorders such as PSC is currently not known. Necrosis involves cell death through autolysis and results in disruption of the integrity of cellular membranes leading to the uncontrolled release of cell contents [15]. Unlike apoptosis, caspase activation does not occur in necrosis. High mobility group box 1 (HMGB1) is a chromatin protein that directly interactions with nucleosomes [16,17]. HMGB1 is involved in the regulation of expression of several genes [18]. HMGB1 is released when a cell undergoes necrosis as the nuclear and cytoplasmic membranes lose their integrity [18,19]. Little HMGB1 is released by cells undergoing apoptosis as it is bound to nucelosomes in apoptotic bodies [20]. HMGB1 is also actively secreted by cells of the innate immune system such as monocytes and macrophages and acts as a proinflammatory cytokine [21,22]. To date, no studies have been conducted to assess serum markers of necrosis in PSC.

Many aspects of the pathophysiology of PSC remain unclear. Although inflammation and fibrosis in PSC are initially focused on the biliary system, the death of hepatocytes is an important component of the disease progression [9]. However, the precise mechanism

through which hepatocyte injury occurs remains unclear. In particular, the relative contributions of apoptosis and necrosis in PSC are not well defined. Therefore, we aimed to determine the degree of hepatocyte apoptosis and necrosis in PSC patients by measuring serum K18 and HMGB1 levels. Additionally, we examined the relationship between these markers and severity of PSC as assessed by liver biochemistries, liver histology, and PSC Mayo Risk Score [23]. In a subgroup of patients that underwent liver biopsy, we also assessed for apoptosis in the liver tissue using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

MATERIALS AND METHODS

Study Cohort

This study consisted of 37 patients with well-characterized PSC, who had not undergone liver transplantation and 39 control subjects. Blood samples from PSC patients were collected as part of Indiana University-Purdue University at Indianapolis Institutional Review Board approved protocol with an informed consent. All samples were obtained over a two year period at the Indiana University Hepatology Clinic, Indianapolis, Indiana. The severity of PSC was assessed biochemically with serum hepatic panel [total bilirubin, alkaline phosphatase (AlkP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin from the day of enrollment], histologically (METAVIR fibrosis score on liver biopsy), and Mayo PSC Risk Score based on the revised natural history model for primary sclerosing cholangitis [23]. Blood samples of 39 blood donors were obtained from Indiana blood bank (Indianapolis, Indiana) to serve as control subjects. These blood donors had (a) normal liver biochemistries, (b) negative serologic test for hepatitis B and C, (c) no significant co-morbidities, and (d) reported no alcohol consumption.

Measurement of caspase-generated K-18 Fragments in serum

Biosamples used in the current study were processed similarly to our previous published protocol [24]. M30, which is caspase-cleaved K18 fragment, a specific marker for apoptosis, were determined using M30-Apoptosense ELISA, per the manufacturer's instructions (Peviva AB, Bromma, Sweden) [24]. Similarly, HMGB1 concentration in serum was measured by ELISA following the manufacturer's protocol (IBL International, Hamburg, Germany). A serum aliquot of 25uL was used for the purpose of this study and absorbance was determined at 450nm with the Vmax Kinetic Microplate reader by Molecular Devices M2 (Sunnyvale, CA). Soft-max Pro software (version 6.2; Sunnyvale, CA) accompanying the microplate reader was used to generate the standard curve and determine the HMGB1 values.

Quantification of hepatocyte apoptosis (through TUNEL assay)

TdT-mediated dUTP-X nick end labeling (TUNEL) assay was performed on a paraffin-embedded sections using the Fluorescein In Situ Cell Death Detection Kit, POD combined with DAB substrate per the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Hepatocyte apoptosis in liver sections was determined by counting the number of TUNEL positive cells in twenty representative 40x microscopic fields across the section and the total number of cells per field was determined by counting the number of nuclei present

in a representative quadrant and multiplying by four. The apoptotic index was calculated as the number of TUNEL positive cells per 400 cells.

Statistical analysis

Descriptive statistics such as means, standard deviation (SD), ranges and percentages were used to characterize the study patients. Comparisons among PSC and control groups were made by the use of non-parametric Mann-Whitney test for the continuous and χ^2 test for the categorical variables. Pearson's correlation coefficients were used to determine the degree of concordance between any two variables. Correlations were considered significant at the < 0.05 level (2 tailed). Quality control procedures, database management, and statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY).

RESULTS

The demographics, body mass index, and liver biochemistries of the PSC and control group are shown in Table 1. The study cohort was 67% male and 93% Caucasian. The mean age of the PSC cohort was slight older than the control group (51.0 ± 2.2 vs. 48.5 ± 13.4 years), but the difference was not statistically significant. Ulcerative colitis (UC) was present in 62% (n=23) of the PSC patients. As expected the liver biochemistries were all significantly elevated in PSC patients compared to the control subjects (p-val = <0.001). Serum K18 levels were significantly elevated in the PSC patients compared to the control (217.4 ± 78.1 vs. 157.0 ± 58.2 U/L, p-val = 0.001) (Table 1, Figure 1A). On the other hand, serum HMGB1 levels were not different between PSC and controls groups (5.38 ± 2.99 vs. 6.28 ± 2.85 ng/mL, p-val = 0.15) (Table 1, Figure 1B). There was no significant correlation between serum levels of K18 and HMGB1 (r = −0.08, p-val =0.6).

Analysis to examine the relationship between serum K18 and liver biochemistries in the PSC cohort showed that serum K18 levels significantly correlated with AST (r = 0.4, p-val = 0.016), Alk P (r = 0.5, p-val = 0.001), total bilirubin (r = 0.6, p-val =<0.001), and albumin (r = −0.4, p-val = 0.02) but not with ALT. Subgroup analysis in the PSC group between patients with and without ulcerative colitis showed elevated but not statistically significant serum K18 levels (234.4 ± 90.9 vs. 189.5 ± 68.4 U/L, p-val = 0.098) in those with UC. There were no significant differences in the liver biochemistries. In contrast, serum HMGB1 levels were significantly lower in PSC patients with UC compared to those without (4.5 ± 2.8 vs. 6.8 ± 2.9 ng/mL, p-val = 0.025).

To determine if the elevated K18 levels in the PSC cohort were due to apoptosis in the liver, we directly quantified apoptosis in the sections through TUNEL assay in patients who had previously undergone liver biopsy (n=16). The level of apoptosis present on the liver biopsy as assessed by the apoptotic index correlated with serum K18 levels (r=0.8, p-val <0.001) (Figure 2) but not with serum HMGB1 levels (r = 0.2, p-val = 0.4). Microscopic examination revealed the presence of apoptosis in hepatocytes and not in the biliary epithelium. There was no obvious zonality to the apoptosis. Histological data by liver biopsy with various stages of fibrosis [METAVIR classification: F0 (n=4), F1 (n=5), F2 (n=3), F3 (n=8), and F4/cirrhosis (n=5)] was available in a subgroup of (n=25) PSC patients. Serum K18 levels were not significantly different among various degrees of fibrosis (p-val = 0.3).

Lastly, we examined the correlation between serum K18 levels and the Mayo PSC Risk Score, a predictive model for patient survival that includes the variables of age, total bilirubin, albumin, AST, and history of variceal bleeding [23]. There was a strong negative correlation between K18 levels and Mayo PSC Risk Score (r=0.40, p-val = 0.015), i.e., higher serum K18 levels were associated with lower predicted survival.

DISCUSSION

Lack of effective therapies for PSC at the current time necessitate a better understanding of the mechanisms involved in the pathogenesis and progression of the disease. In the present study, increased serum K18 levels without a concomitant increase in HMGB1 levels support the important role of hepatocyte apoptosis in disease progression in individuals with PSC. Furthermore, the close correlation of serum K18 levels with several serum biochemical markers of liver disease severity and hepatocyte apoptotic index further supports the critical role of hepatocyte apoptosis in progression of disease in patients with PSC. It raises the possibility for use of K18 as a potential biomarker for disease severity in patients with PSC. Alternatively, it may even serve as a biomarker to assess response to therapy.

In the current study, the presence of apoptosis in the hepatocytes with no involvement of biliary epithelium suggests that the initial event of biliary structure from mechanisms that currently remain unclear result in hepatocyte cell death possibly through the inflammatory crosstalk between the reactive cholangiocytes and inflammatory cells promoting diffuse hepatocyte injury through apoptotic pathways. Liver-targeted caspase inhibitors could be an attractive treatment option for these patients and may be safely tolerated even in those with concomitant inflammatory bowel disease. Lastly, liver biopsy has a limited role in the evaluation and management of PSC due to asymmetrical involvement of biliary tract and heterogeneous fibrosis. In fact, Mayo Clinic Risk Score, the natural history model for prediction of survival in PSC patients was developed without the use of histologic staging [23]. Lack of relationship between serum K18 level and stage of fibrosis in the current study is therefore not a surprise.

A certain aspect of the current study may need further discussion. Keratin 18 is highly expressed in cholangiocytes but more moderately expressed in hepatocytes. Furthermore, it is also widely expressed throughout the intestine (http://www.proteinatlas.org/search/keratin+18). Thus, it is possible that the elevated K18 could also originate from damaged intestinal epithelial cells. The higher levels of K18 present in serum of PSC+UC patients as compared to PSC-only patients (probably significant if larger cohorts would have been analyzed) could suggest that serum K18 instead partly originates from the intestine.

In summary, the current study suggests that hepatocyte apoptosis plays a predominant role in the pathogenesis of liver injury and disease progression in PSC. In future, longitudinal studies need to be performed with serial K18 measurements and examine its biomarker potential for disease progression or therapeutic response. Additional studies that would examine bile acid composition and cross-talk between reactive cholangiocytes and inflammatory cells would be very informative.

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Abbreviations

ALT	Alanine aminotransferase
Alk P	Alkaline phosphatase
AST	Aspartate aminotransferase
HMGB1	High-mobility group protein B1
K18	Cytokeratin-18 fragment
PSC	Primary sclerosing cholangitis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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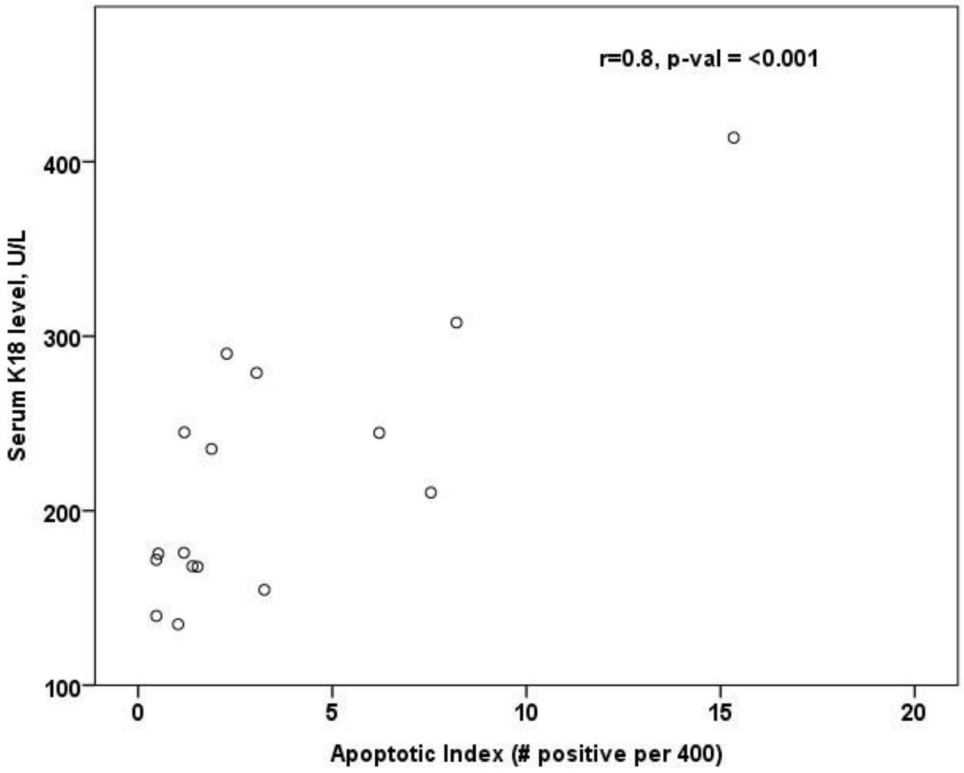

Figure 1.

Serum levels of K18 and HMGB1 in PSC (n=37) and control (n=39) groups. (A) Serum K18 levels were significantly elevated in PSC group when compared to control (217.4 ± 78.1 vs. 157.0 ± 58.2 U/L, *p-val = 0.001). (B) Serum HMGB1 levels were not significantly different between the two groups (p-val = 0.15).

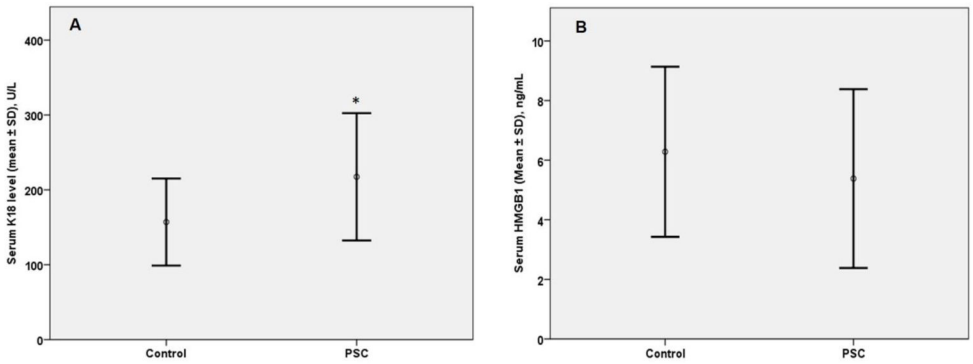

Figure 2.

Serum K18 levels correlated with the degree of apoptosis as quantified in the liver biopsy section through TUNEL assay (r=0.9, p-val = <0.001).

Table 1

Demographics, liver biochemistries and serum markers of hepatocyte injury in patients with primary sclerosing cholangitis (PSC) and control subjects from healthy blood bank donors. All values are presented in mean ± standard deviation unless otherwise specified.

	PSC (N=37)	Controls (N=39)	p-value
Demographics			
Age (years)	51.0 ± 2.2	49 ± 13	0.43
Male (%)	57	77	0.1
Caucasian (%)	89	97	0.3
BMI (kg/m2)	28.3 ± 5.9	30.7 ± 6.6	0.19
Ulcerative colitis (%)	62%	Not applicable	
Liver biochemistries			
Total Bilirubin (mg/dL)	2.07 ± 2.34	0.53 ± 0.19	<0.001
AST (U/L)	63 ± 37	24 ± 4	<0.001
ALT (U/L)	58 ± 35	17 ± 5	<0.001
Alkaline phosphatase (U/L)	306 ± 216	62 ± 12	<0.001
Markers of hepatocyte injury			
Keratin 18 fragments (U/L)	217.39 ± 78.14	156.99 ± 58.17	0.001
HMGB1 (ng/mL)	5.38 ± 2.99	6.28 ± 2.85	0.15