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Serum acid sphingomyelinase is upregulated in chronic hepatitis C infection and non alcoholic fatty liver disease☆,☆☆

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ABSTRACT

Sphingolipids constitute bioactive molecules with functional implications in homeostasis and pathogenesis of various diseases. However, the role of sphingolipids as possible disease biomarkers in chronic liver disease remains largely unexplored. In the present study we used mass spectrometry and spectrofluorometry methods in order to quantify various sphingolipid metabolites and also assess the activity of an important corresponding regulating enzyme in the serum of 72 healthy volunteers as compared to 69 patients with non-alcoholic fatty liver disease and 69 patients with chronic hepatitis C virus infection. Our results reveal a significant upregulation of acid sphingomyelinase in the serum of patients with chronic liver disease as compared to healthy individuals ($p < 0.001$). Especially in chronic hepatitis C infection acid sphingomyelinase activity correlated significantly with markers of hepatic injury ($r = 0.312$, $p = 0.009$) and showed a high discriminative power. Accumulation of various (dihydro-) ceramide species was identified in the serum of patients with non-alcoholic fatty liver disease ($p < 0.001$) and correlated significantly to cholesterol ($r = 0.448$, $p < 0.001$) but showed a significant accumulation in patients with normal cholesterol values as well ($p < 0.001$). Sphingosine, a further bioactive metabolite, was also upregulated in chronic liver disease ($p < 0.001$). However, no significant correlation to markers of hepatic injury was identified. *Conclusion*: Chronic hepatitis C virus infection and non-alcoholic fatty liver disease induce a significant upregulation of serum acid sphingomyelinase which appears as a novel biomarker in chronic hepatopathies. Further studies are required to elucidate the potential of the sphingolipid signaling pathway as putative therapeutic target in chronic liver disease.

1. Introduction

Sphingolipids (SLs) constitute bioactive lipid molecules with pleiotropic effects on cellular functions and consequently on the pathophysiology and pathogenesis of various diseases such as cancer, atherosclerosis, infections, obesity, insulin resistance and storage diseases denoted as sphingolipidoses [1]. The assessment of SL-concentrations in human serum, also termed serum sphingolipidomics, represents a quickly evolving field of translational medical research [2,3]. With recent experimental evidence proposing a critical role for SLs in the pathophysiology of liver diseases [4], the evaluation of potential SL biomarkers in chronic liver disease seems quite intriguing.

Ongoing basic research studies reveal a central role for bioactive SL metabolites in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) [5–8] and chronic hepatitis C virus (HCV) infection [9–13]. Particularly ceramide, the backbone of complex SLs and important second messenger inducing cell death, inflammation and oxidative

Abbreviations: SL, sphingolipid; NAFLD, non-alcoholic fatty liver disease; HCV, hepatitis c virus; NASH, non-alcoholic steatohepatitis; ASM, acid sphingomyelinase; S1P, sphingosine1-phosphate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γGT, gamma-glutamyl-transferase; AP, alkaline phosphatase; LDL, low-density lipoprotein; BMI, body mass index; HOMA, homeostatic model assessment; DHC, dihydroceramide; ROC, receiver operating characteristic; AUC, area under the curve; PKCδ, protein kinase Cδ; Suppl., Supplementary

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stress [14], has been linked both to the development of NAFLD [6] as well as to the progression to non-alcoholic steatohepatitis (NASH) by inhibiting insulin signaling [15]. Furthermore, the generation of ceramide within biological membranes alters the biophysical properties of these membranes [16,17] thus amongst others affecting both entry of HCV into the host cell [12] as well as formation of the HCV replication complex [9,10]. Additionally, sphingomyelin, the most abundant SL, has been shown to activate the NS5B polymerase of HCV in a genotype specific manner and thus to promote HCV replication [18]. Thereby, the hydrolysis of sphingomyelin to ceramide, especially via action of the acid sphingomyelinase (ASM), is able to regulate the hepatocellular susceptibility to various stimuli and thus to critically influence hepatocellular apoptosis [4,19], an integral process in the pathogenesis of both HCV-infection and NAFLD [20–22].

Serum or plasma ceramides constitute evident disease biomarkers in neurodegenerative disorders [23], obesity and diabetes mellitus [24] as well as in acute phase reactions [25], whereas their functional antagonist sphingosine1-phosphate (S1P), which promotes proliferation and cell growth, is implicated as a novel biomarker in various cancer diseases [26,27]. Similar biomarker properties such as prediction of the progression and severity of diseases like major depression [28], acute and chronic alcohol abuse [29], chronic heart failure [30], severe sepsis [31] and type 2 diabetes [32] have been attributed to ASM as well. However, despite the fact that experimental evidence has already highlighted the ASM-mediated generation of ceramide as an important mechanism mediating liver injury [19,33–37], the role of SL metabolites, especially ceramide and ASM, as potential biomarkers in chronic liver disease has not been examined so far.

Purpose of the present study is therefore to evaluate SL parameters in the serum of patients with NAFLD and chronic HCV infection compared to healthy controls as possible biomarkers in chronic liver disease. With the background of ceramide generation being a metabolic hub in SL metabolism we assessed the activity of ASM, an essential regulator of ceramide levels, in the serum of patients with chronic liver disease as compared to healthy controls. Beside the determination of ASM activity we additionally tried to address as far as possible the comprehensive alterations of various SL metabolites in the serum of healthy volunteers, of patients with NAFLD and of patients with chronic HCV infection in order to provide evidence for serum sphingolipidomic modifications in chronic liver disease.

2. Patients and methods

2.1. Patients

Serum samples were collected from patients with NAFLD ($n = 69$) and from patients with chronic HCV infection ($n = 69$) advised at the hepatological outpatient clinic of the Goethe University Hospital. Patients with liver cirrhosis were excluded from the NAFLD and HCV patient cohorts. At the time point of serum sample collection none of the patients included in the current analysis was diagnosed with hepatocellular carcinoma. Additionally, serum samples from healthy volunteers ($n = 72$) with no evidence of chronic liver disease (students, paramedical and medical staff of the Goethe University Hospital, screened regularly by the in-house medical service) were collected as well, in order to evaluate alterations of SL parameters within healthy subjects. Both patient groups (HCV and NAFLD) were age-matched, whereas the control group of healthy volunteers was overall younger than the patient groups. Standard biochemical parameters were measured at the central laboratory of the Frankfurt University Hospital. Viral load was available in 61 out of 69 HCV patients (88.4%), whereas viral genotype was available for all HCV patients. NAFLD was diagnosed in patients with unknown hepatopathy and hepatic steatosis in the liver ultrasonography after exclusion of a viral, autoimmune, alcohol-induced and metabolic liver disease. The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee. All patients had signed a written informed consent prior to study inclusion.

2.2. Statistical analysis

Calculations were made with GraphPad Prism v5.01 for Windows (GraphPad Software, San Diego, CA) and by using the BiAS software for windows (version 9.16, Epsilon-Verlag, Darmstadt, Germany). Statistical comparisons were carried out using the non-parametric Mann Whitney-U and Kruskal–Wallis tests in order to determine statistically significant differences among patient groups. The data are expressed as Means ± Standard Error of the Mean (SEM) unless otherwise specified. The level of significance is set at $\alpha = 00.05$ representing the 95% confidence interval. Statistically significant differences are indicated in the corresponding Figures: "*" = $p < 0.05$, "**" = $p < 0.01$, "***" = $p < 0.001$. The correlation coefficient rho was calculated by using the Spearman correlation provided in BiAS software. Receiver operating characteristic (ROC) curves and area under the curve (AUC) values were calculated by the BiAS software as well.

Details on additional materials and methods are provided in the supplementary material.

3. Results

3.1. Patient characteristics

We assessed the activity of ASM and the concentrations of various SL parameters in the serum of 72 healthy individuals, 69 patients with NAFLD and 69 patients with chronic HCV infection. Patients with chronic liver disease were overall older and showed significant higher values of hepatic injury compared to healthy individuals as shown in Table 1. In contrast to healthy individuals and NAFLD patients, most of the HCV patients were male. However, no significant differences concerning gender distribution within the three groups were observed (Table 1).

3.2. ASM activity is upregulated in serum of patients with chronic liver disease

Clinical relevance has been attributed to enhanced peripheral ASM activity by several studies. Therefore we assessed the activity of the enzyme in serum samples of NAFLD, HCV patients and healthy individuals. Our data show a significant upregulation of mean peripheral ASM activity (fmol/h/μl) in chronic HCV infection (628.8 ± 38.1) and in patients with NAFLD (408.9 ± 21.2) as compared to healthy individuals (179.9 ± 7.0) (for all comparisons $p < 0.001$) (Fig. 1A). Furthermore, in patients with chronic liver disease ASM activity showed a significant increase in HCV infection as compared to NAFLD ($p < 0.001$). Since total protein levels showed significant differences among patient groups (Table 1) we normalized ASM activity values to total protein concentrations. HCV and NAFLD patients still showed elevated mean ASM activities per g/dl protein (87.7 ± 5.5 and 56.2 ± 2.9 respectively) when compared to healthy individuals (25.9 ± 1.1) (for all comparisons $p < 0.001$) (Fig. 1B).

3.3. Elevated serum (dihydro-)ceramide species in NAFLD

Beside ASM activity, serum concentrations of various ceramide, the direct product of ASM catalysis, and dihydro-ceramide (DHC) species were assessed as well, in order to evaluate corresponding variations of SL metabolites potentially caused by the observed upregulation of ASM activity in chronic liver disease. DHCs constitute ceramide precursors and are produced either anabolically via the de novo synthetic pathway of sphingolipid metabolism or catabolically by the hydrolysis of dihydro-sphingomyelin (Suppl. Fig. 6). Patients with NAFLD showed