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Original Article

Hepatic steatosis causes induction of the chemokine RANTES in the absence of significant hepatic inflammation

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Abstract: Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum ranging from simple steatosis to cirrhosis. Hepatocellular lipid accumulation is a hallmark of both nonalcoholic steatosis and steatohepatitis (NASH). The latter develops upon pro-inflammatory cell infiltration and is widely considered as the first relevant pathophysiological step in NAFLD-progression. The chemokine CCL5/RANTES plays an important role in the progression of hepatic inflammation and fibrosis. We here aimed to investigate its expression in NAFLD. Incubation of primary human hepatocytes with palmitic acid induced a dose-dependent lipid accumulation, and corresponding dose-dependent RANTES induction *in vitro*. Furthermore, we observed significantly elevated hepatic RANTES expression in a dietary model of NAFLD, in which mice were fed a high-fat diet for 12 weeks. This diet induced significant hepatic steatosis but only minimal inflammation. In contrast to the liver, RANTES expression was not induced in visceral adipose tissue of the group fed with high-fat diet. Finally, RANTES serum levels were elevated in patients with ultrasound-diagnosed NAFLD. In conclusion, our data indicate hepatocytes as cellular source of elevated hepatic as well as circulating RANTES levels in response to hepatic steatosis. Noteworthy, upregulation of RANTES in response to lipid accumulation occurs in the absence of relevant inflammation, which further indicates that hepatic steatosis *per se* has pathophysiological relevance and should not be considered as benign.

Keywords: Hepatic steatosis, liver cirrhosis, nonalcoholic steatosis, steatohepatitis, chemokine CCL5, RANTES, inflammation, nonalcoholic fatty liver disease (NAFLD)

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition with excessive fat accumulation in hepatocytes [1]. NAFLD is often classified as primary and secondary according to the underlying etiology [2]. Primary NAFLD generally refers to fatty liver associated with features of the metabolic syndrome [3], and in fact, NAFLD may be considered as hepatic manifestation of the metabolic syndrome [4].

Recent studies on the development and progression of NAFLD emphasize the pathophysiological role of cytokines and chemokines besides that of insulin resistance, oxidative stress and lipid peroxidation [5]. Chemokines represent a large superfamily of proteins that possess diverse biologic activities [6]. Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES, also known as CCL5) is a CC beta-chemokine with strong chemoattractant activity for T lymphocytes and monocytes [7]. It has been implicated in the pathogenesis of numerous diseases including cancer [8], neurological disorders [9], asthma [10], autoimmune diseases such as rheumatoid arthritis [11] and renal diseases [12].

Increased (hepatic) RANTES expression has been reported in patients with chronic viral hepatitis [13,14], and a comprehensive study by Seki and colleagues [15] has shown that (increased expression of) RANTES promotes

hepatic inflammation and fibrosis in the bile duct ligation model and in a model of chronic toxic liver injury by application of tetracarbonchloride. Furthermore, it has been reported that obesity promotes chemokine expression in patients with chronic hepatitis C infection [16], and increased hepatic RANTES expression has been observed in a murine NASH-model [17] and in obese patients [18].

Based on the hypothesis that increased hepatic RANTES expression is an early event in NAFLD and a critical factor influencing its progression the aim of the present study was to analyze the effect of hepatocellular lipid accumulation on RANTES expression in the absence of significant hepatic inflammation.

Materials and methods

Primary human hepatocytes and in vitro model of hepatocellular lipid accumulation

Isolation and culture of primary human hepatocytes (PHH) were performed as described [19]. Human liver tissue for cell isolation was obtained according to the guidelines of the charitable state-controlled foundation HTCR with the patient's informed consent. Hepatocellular lipid accumulation was induced as described [20]. Briefly, a 10 mM palmitate/1% bovine serum albumin (BSA) stock solution was obtained by complexing the appropriate amount of palmitate to BSA. PHH were grown in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 mg/ml), L-glutamine (300 mg/ml), 0.2% FCS and palmitate for 24h in different concentrations as indicated. FFA-free-BSA-treated cells served as controls.

Intracellular triglyceride assay

Total triglycerides were extracted using the method of Bligh and Dyer with slight modifications [21,22] and quantified by the triglyceride determination kit (GPO) (Sigma, Deisenhofen, Germany) as described [20].

Murine model of hepatic steatosis

Male BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at 6 weeks of age and housed under standard conditions. After 1 week of acclimatization mice were divided into two groups (n=6 each) and fed either with control diet or a high fat diet (HFD) containing 30% lard. All chows were prepared by Ssniff (Soest, Germany). After 12 weeks feeding animals were sacrificed. Liver tissue and epididymal fat were immediately snap-frozen and stored at -80°C for subsequent analysis.

Human serum samples of patients with ultrasound-diagnosed fatty liver and control patients

Serum samples were obtained from randomly selected patients recruited from the referrals of the ultrasound (US) centre at our clinic from January 2008 to January 2009. According to the results of a standardized ultrasound examination participants were divided into 2 groups – a control group with normal US liver appearance and a group of patients with US-diagnosed fatty liver. The following exclusion criteria were applied: history of malignancy of any kind, chronic hepatobiliary diseases, use of medication known to affect hepatic steatosis (e.g. glucocorticoids, anti-estrogens), inflammatory bowel disease, human immunodeficiency virus infection, drug or alcohol abuse (more than 20 g/day), familial hyperlipidemia, and acute medical emergencies. Further, patients with clinical or laboratory signs of systemic inflammation were excluded to omit possible confounding effect on serum RANTES.

After collection serum samples were immediately snap frozen and stored at -80°C. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee.

RANTES expression analysis

Isolation of total cellular RNA from cultured cells and tissues and reverse transcription were performed as described [23]. Quantitative real time -PCR was performed with specific sets of primers applying LightCycler technology (Roche, Mannheim, Germany) as described [24] and specific human and mouse RANTES Primer assays (Qiagen, Hilden, Germany).

Analysis of RANTES serum levels

RANTES in human sera was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) using the human CCL5 DuoSet ELISA development kit (R&D Systems Inc., MN, USA, catalog number DY278).

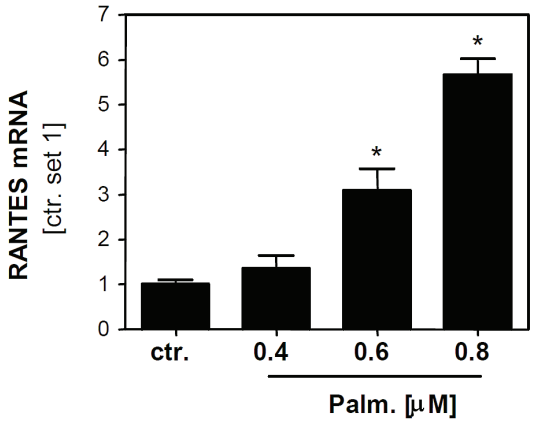

Figure 1. RANTES expression in an *in vitro* model of hepatocellular lipid accumulation. Primary human hepatocytes were incubated with 0.4 µM, 0.6 µM and 0.8 µM palmitate (Palm.) for 24 h, which leads to a dose dependent intracellular lipid accumulation as shown before [20]. Subsequently, RNA was isolated, reverse transcribed and RANTES mRNA expression was analyzed by quantitative PCR. (*p<0.05 compared to control (ctr.)).

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, IL, USA) and GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA). Results are expressed as means ± standard error (range). Comparisons between groups were made using one way analysis of variance (with Bonferroni correction for multiple comparisons). P-values <0.05 were considered as statistically significant.

Results

Hepatocellular lipid accumulation induced RANTES expression in vitro

Recently, we have described an *in vitro* model of hepatocellular lipid accumulation [20]. Incubation of primary human hepatocytes (PHH) with palmitic acid led to a dose dependent increase of hepatocellular free fatty acid and triglyceride levels [20]. Interestingly, this lipid accumulation led to a significant induction of RANTES mRNA expression in PHH (**Figure 1**).

Increased hepatic RANTES expression in steatotic murine livers

Next, we analyzed RANTES expression in livers of mice fed a high-fat diet (HFD) [25]. Feeding this diet led to significant hepatic lipid accumulation (**Figure 2A**). In contrast mice in the HFD-group had normal levels serum transaminases (**Figure 2B**) and revealed only slightly elevated TNF mRNA expression (**Figure 2C**).

Activated hepatic stellate cells (HSC) are the cellular source of excessive extracellular matrix

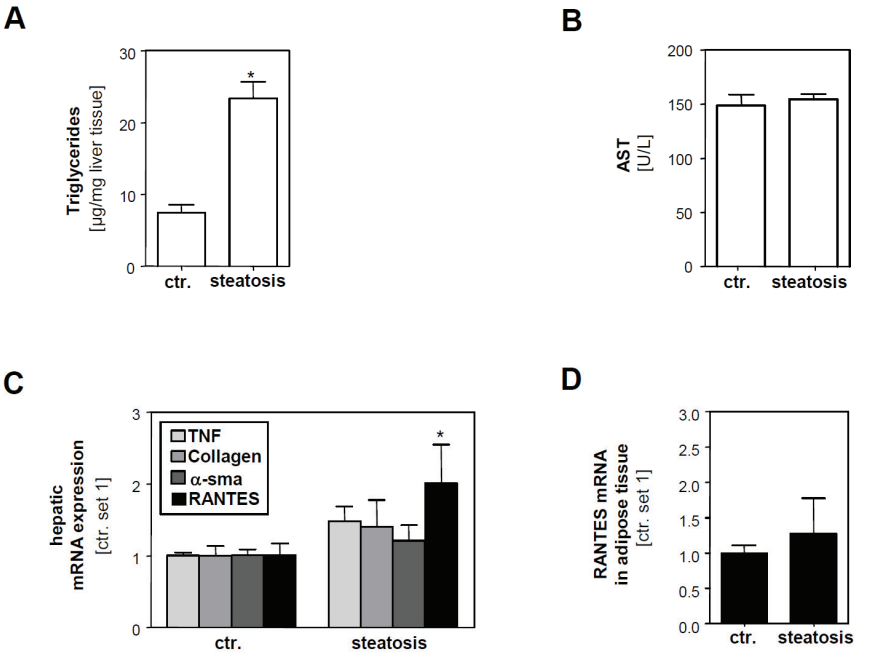

Figure 2. RANTES expression in the liver and in visceral fat in a murine model of NAFLD. Mice were fed a high fat diet leading to hepatic steatosis. Mice receiving standard chow served as control (ctr.). **(A)** Assessment of hepatic triglyceride content. (**B**) Serum GOT levels. Analysis of **(C)** hepatic TNF, collagen I, α-smooth muscle actin (α-sma), and RANTES mRNA expression, and **(D)** expression of RANTES mRNA in visceral fat by quantitative PCR. (*p<0.05 compared to control).

deposition in chronic liver injury [26], and previous studies have shown increased RANTES expression in activated HSC [27]. However, neither expression of alpha-smooth muscle actin, a sensitive marker of HSC activation, nor collagen I expression was elevated in HFD-fed mice, indicating that there was no relevant activation of HSC in the livers of these animals (**Figure 2C**). However and notably, RANTES expression was significantly increased in mice fed with the HFD (**Figure 2C**).

It has been shown that visceral fat is a significant source of proinflammatory cytokines including RANTES [28,29]. Therefore, we also analyzed RANTES expression in visceral fat but found only minimally increased mRNA expression compared to control mice (**Figure 2D**).

Increased RANTES serum levels in patients with non-alcoholic fatty liver

Recently, we have described a cohort of patients with referral for sonographic examination of the abdomen and ultrasound-diagnosed NAFLD [30]. Analysis of serum levels of RANTES revealed slightly but significantly elevated RANTES levels in patients with ultrasound-diagnosed NAFLD (n=45; mean: 85.9 ng/ml, SEM 3.7 ng/ml,) in comparison to the control group (n=61; mean: 60.1 ng/ml, SEM 2.9 ng/ml; p<0.001), **(Figure 3)**.

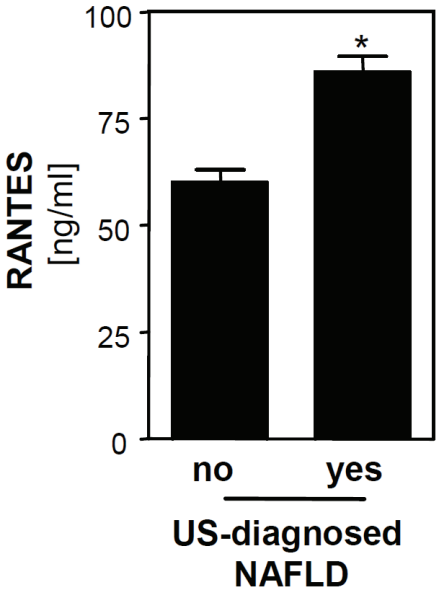

Figure 3. Serum RANTES levels in patients with NAFLD. Serum RANTES levels in patients with ultrasound (US) diagnosed fatty liver compared to a control (ctr.) group of patients with normal sonographic liver appearance. (*p<0.05 compared to control).

Discussion

The chemokine RANTES has been shown to play a central role in the pathogenesis and progression of chronic liver disease, however, present information regarding its expression in nonalcoholic fatty liver disease (NAFLD) is sparse [31].

RANTES has been shown to be expressed by hepatocytes and is up-regulated in response to inflammatory conditions [14;32-34]. Here, we reveal that lipid accumulation did also dose-dependently induce RANTES expression in primary human hepatocytes *in vitro*. Thus, pure hepatocellular steatosis leads to increased hepatic RANTES expression. In line with this, we observed that feeding a high-fat diet induced hepatic RANTES expression in the absence of significant hepatic inflammation or fibrosis. This finding further confirms steatotic hepatocytes but not infiltrating inflammatory cells or activated hepatic stellate cells as main cellular sources of elevated hepatic RANTES expression in high-fat diet fed mice.

Nevertheless, it has to be considered that obesity frequently leads to up-regulation of cytokines as a part of a systemic state of low inflammation not only in the liver but also in (visceral) adipose tissue, which is another important source of circulating cytokines including RANTES [29]. However, in our study, RANTES expression in visceral fat of mice, which were fed with a high-fat diet, was similar to the control group. This suggests that at least under these experimental conditions the fatty liver but not adipose tissue is the source of elevated (circulating) RANTES levels.

Taken together with our *in vitro* data and the known pathophysiological role of RANTES in other chronic liver diseases, this indicates that steatosis-triggered RANTES production by hepatocytes is an early event during the natural course of NAFLD and may be involved, along with other factors/cytokines, in the progression of fatty liver to significant inflammation, e.g. NASH.

Interestingly, patients with US-diagnosed NAFLD had significantly elevated RANTES serum levels

compared to the control group, although this increase was only moderate compared to the induction of RANTES mRNA expression in *in vitro* lipid loaded hepatocytes and murine fatty livers. However, although US examination can be adequately used to assess hepatic steatosis [35,36], it has to be considered that the reliable threshold is approximately 30% fat in the hepatic tissue [37]. Thus, it is likely that a significant number of patients in the control group also had hepatic steatosis below the US-detection level. NAFLD is generally defined by lipid deposition greater than 5-10% of the liver weight [1], and our *in vitro* data indicate that even minimal hepatocellular lipid accumulation leads to increased RANTES levels. Conversely, despite the lack of histological examination in this hospital cohort of randomly selected patients, it can be estimated from epidemiological studies [38], that only a minority of cases had significant hepatic inflammation (e.g. criteria for NASH), which may have caused elevated RANTES levels.

In summary, our study indicates that hepatic steatosis causes an up-regulation of hepatic RANTES expression. Noteworthy, increased expression of RANTES in response to hepatocellular lipid accumulation can occur in the absence of relevant hepatic inflammation. This finding further indicates that hepatic steatosis *per se* has pathophysiological relevance and should not be considered as benign.

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