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Macrophages, TGF-β1 expression and iron deposition in development of NASH

Research Article

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Abstract: A wide range of molecular markers and different types of cells in liver are possible factors for progression of non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) development of liver fibrosis. We investigated biopsies from 57 patients with NASH. The material was obtained from livers and was proceed immunohistochemistry antibodies against CD68 and TGF-beta 1. In addition,biopsies were evaluated for iron content. Macrophages/-positive/could be found in all 57 cases. The number of macrophages in the sinusoids correlated with the degreeof portal fibrosis:64.% ofthe patients with mild or intensive fibrosis had high infiltration with CD68-positive cells, while 100% ofthe patients without fibrosis hadlow infiltration ($\chi 2=8.56$; $p=0.003$). In specimens we ,69.% of patientswith different degreeof fibrosis expressed TGF-β1 in their portal tracts, and 100% ofpatients without fibrosis did demonstrate expression of the protein ($\chi 2=23.7$; $p<0.001$). Hepatic iron was found in 100% (9) of patients with intensive fibrosis vs. 10.3% of the patients mild fibrosis ($\chi 2=23.4$; $p<0.001$). Our results suggest that the macrophages and macrophage-derived TGF-beta1 are the major factors responsible for development of fibrosis and progression of chronic liver disease.

Keywords: *NASH • CD68 • TGF-beta1 • Iron*

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by accumulation of fat in the hepatocytes (steatosis); this condition is frequent and probably completely benign [1]. If steatosis is associated with inflammation, Mallory bodies and signs of impending cell death such as ballooning, the entity is termed non-alcoholic steatohepatitis (NASH). NASH can lead to fibrosis, cirrhosis and even hepatocellular cancer [2]. Steatosis was once believed to be a benign condition, with rare progression to chronic liver disease; however, steatohepatitis may progress to liver fibrosis and cirrhosis, and may result in liver-related morbidity and mortality. Fibrosis or cirrhosis in the liver is present in 15%–50% of patients with NASH [3]. Approximately 30% of patients with fibrosis develop cirrhosis after 10 years.

Nonalcoholic steatohepatitis (NASH) was described by Ludwig mainly in obese, middle-aged women, often associated with diabetes mellitus and hyperlipidemia. In recent years, NASH has also been found in with male, nonobese, nondiabetic patients and is associated with with liver iron overload; that led to the hypothesis that

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iron plays a role in NASH pathogenesis. Increased ferritin with normal transferrin saturation is frequently found in patients with fatty liver, but reflects iron overload only in those patients in whom it persists despite an appropriate diet [4]. Insulin resistant hepatic iron overload (IR-HIO) is a newly described condition of hepatic iron overload, characterized by hyperferritinemia with normal or slightly increased transferrin saturation in the absence of hemochromatotic gene mutations [5]. Although patients with IR-HIO have a high prevalence of insulin resistance-related metabolic disorders, the relationship of IR-HIO and NASH is unclear. Two characteristics allow differentiation of IR-HIO from genetic haemochromatosis: iron overload is heterogeneous from one hepatocyte to another in the periportal area, and sinusoidal iron is distributed throughout the lobule. In IR-HIO, fibrosis develops at a much lower hepatic iron burden than in genetic haemochromatosis; sinusoidal iron, steatosis and inflammation could represent the histological mark of activity and progression of liver disease in IR-HIO [6].

A wide range of molecular markers and different types of cells in liver could be prognostic markers for progression of liver fibrosis and development of NAFLD respectively NASH. A central role in fibrogenesis has been assigned to transforming growth factor-b1 (TGF-b1), which is a particularly well-studied cytokine and widely regarded as a pro-fibrogenic agent in liver injury, particularly after chronic liver injury [7]. High levels of TGF-b1 are often found in hepatic fibrosis, and it has been implicated as a mediator of fibrosis in many liver diseases [8]. The multiple biological actions of TGF-b1, which involve in a variety of biological processes including development, cell growth, differentiation, cell adhesion, migration, extracellular matrix deposition and the immune response, contribute to the regulation of the production, degradation and accumulation of extracellular matrix proteins in a direct or indirect manner. Fibrotic pathologies are associated with increased levels of TGF-b1 that initially recruit inflammatory cells and fibroblasts into an area of injury and then stimulate these cells to produce cytokines and extracellular matrix.

The aim of our study was to investigate the significance of macrophage infiltration, TGF-beta expression and iron deposition in development of NASH patients.

Table 1. Demographic, laboratory and histological features of the patients.

Parameter Clinical and histological data	Number (%) (n=57)
Age (years)	
median	60,6
(range)	(28-86)
Sex	
Male	27 (47,4)
Female	30 (52,6)
Hepatosteatosis	
weak	30 (52,6)
strong	27 (47,4)
Fibrosis	
No	18 (31,6)
Mild	30 (52,6)
Intensive	9 (15,8)
Portal inflamation	
weak	44 (77,2)
strong	13 (22,8)
Iron deposition	
Yes	13 (22,8)
No	44 (77,2)
AST	41,15 (11-100)
ALT	37,35 (12-70)

2. Materials and methods

2.1. Tissue samples and patients characteristics

Specimens were obtained from 57 patients between 2008 and 2011. The patients comprised 27 males and 30 females, aged 28 to 86 years (mean 60.6 years). The pathologic diagnosis of NASH was based on the following: a liver biopsy showing the presence of steatosis (>10%), lobular inflammation and hepatocellular degeneration irrespective of the presence of fibrosis and appropriate exclusion of other liver diseases (alcohol abuse, hepatitis B surface antigen positivity, autoimmune hepatitis or HCV infection).

Biopsies were evaluated for grade and stage according to Ishak, and for iron content on sections stained with Perl's Prussian-blue. Steatohepatitis was graded based on the degree of macrovesicular steatosis, mixed lobular inflammation and hepatocyte ballooning using a composite NAFLD activity score (NASH > 5) [9]. The presence of portal fibrosis was noted and scored as none, mild or intensive.

The main clinical and histological data are presented in Table 1. Informed consent was obtained from all patients.

2.2. Immunohistochemistry

For immunohistochemical staining, paraffin blocks were prepared using tumor tissues from the periphery of the tumor adjacent to the normal tissues. Paraffin sections 5 μm thick were dewaxed in two xylenes and

were rehydrated in ethanol. Then they were washed in 0.1 M phosphate buffered saline (PBS), pH 7.4, incubated in 1.2% hydrogen peroxide in methanol for 30 min, and rinsed in 0.1 M PBS, pH 7.4, for 15 min. The immunohistochemical reactions were carried out using the following: monoclonal mouse anti-human CD68 antibody (N1576, DAKO) in 1:50 dilution and rabbit anti-human TGF-β1 antibody (sc-146, Santa Cruz Biotechnology, USA) in a dilution 1:50. After washing three times in PBS, the slides were incubated with the DAKO-REAL™ En-Vision™ detection system (DAKO) for 60 min, then visualized with 3,3'-diaminobenzidine and counterstained with hematoxylin. We used tissues known to exhibit high levels of marker for the positive control and as the negative control, the primary antibody replaced with PBS.

2.3. Statistical analysis

The SPSS 16.0 program for Windows was used for statistical analysis. The descriptive statistical tests, including the mean, standard deviation and median, were calculated according to standard methods. The frequency of distribution of immunohistochemical staining and the clinicopathological parameters in 2x2 contingency tables were analyzed by the χ^2-test. For all statistical analyses, $p<0.05$ was considered to be statistically significant.

3. Results

We found infiltration of liver tissue by CD68-positive cells in all specimens studied (Figure 1). Patients were divided into groups according to high and low infiltration with these cells in liver tissue. CD68-positive cells could be found in all 57 cases, with a median number of 13.5 (range 0.54–96.0) for the portal tracts, and median number of 35.9 (range 0.27–97.02) for the sinusoids.

When comparing the density of cells expressing CD68 between the investigated localizations, no statistically significant differences were found (p=0.144 and p=0.56, respectively; Mann-Whitney U test).

In the next set of analyses, we examined the correlation between the infiltration of CD68-positive cells and clinicopathologic factors. Again, patients were divided into groups with low and high infiltration based on the 75th percentile. There were no associations between the number of CD68-positive cells in portal tracts and all clinicopathological parameters studied (data not down). However, the number of macrophages in the sinusoids correlated with degree of portal fibrosis: 64.1% of the patients with mild or intensive fibrosis had high infiltration with CD68-positive cells, while 100% of the patients without fibrosis had low infiltration ($\chi 2=8.56$; p=0.003).

In our study, 30 (52.6%) of 57 patients displayed portal tract TGF-β1 protein reactivity (Figure 2). TGF-β1

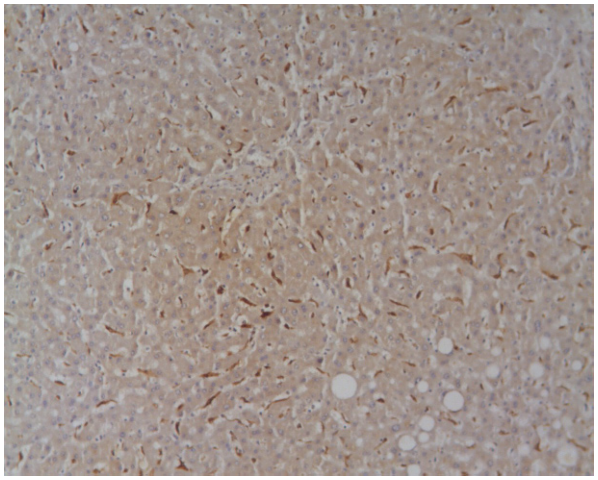

Figure 1. Expression of CD68-positive macrophages in liver lobules and portal tracts /x100/.

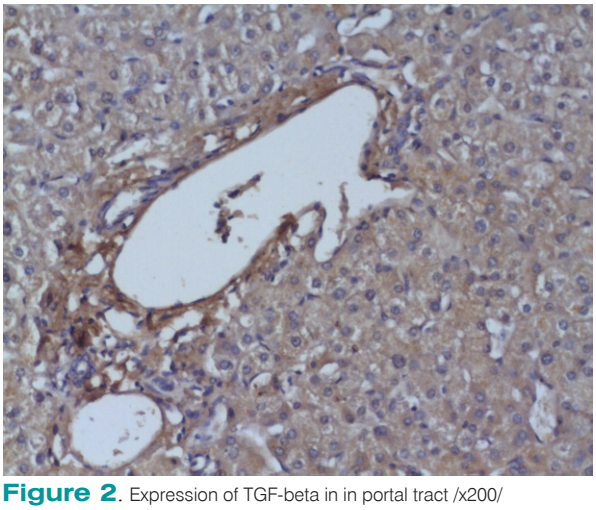

Figure 2. Expression of TGF-beta in in portal tract /x200/

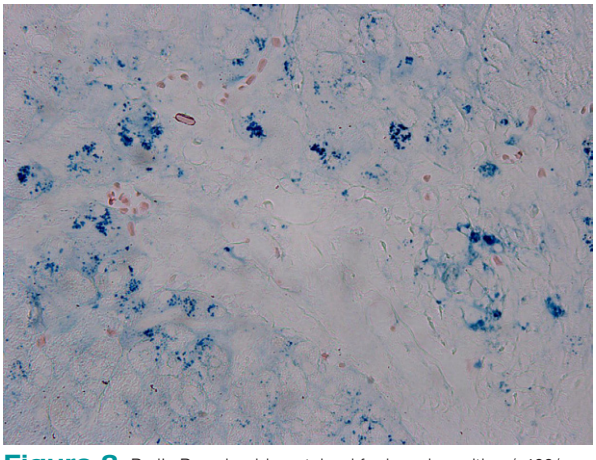

Figure 3. Perl's Prussian-blue-stained for iron deposition /x400/

expression was correlated to a high degree of portal fibrosis. In specimens investigated, we found that 69.2% of patient with different degrees of fibrosis expressed TGF-β1 in their portal tracts, and 100% of patients without fibrosis did not demonstrate expression of the protein ($\chi 2=23.7$; $p<0.001$). There was no correlation between numbers of CD68-positive cells and TGF-β1 expression.

Hepatic iron in sinusoidal cells (Kupffer cells – ever macrophages) was observed in 100% (9) of patients with intensive fibrosis, vs. 10,3% of the patients mild fibrosis ($\chi 2=23.4$; $p<0.001$) (Figure 3). There was no correlation between iron deposition and other clinicomorphological factors.

4. Discussion

Our data suggest that expression of TGF-beta 1 correlates to a high degree of portal fibrosis. In addition, the number of macrophages (CD68-positive cells) was related to this expression and again correlated with mild or intensive fibrosis in patients of the group.

Interest position in development of liver injury, fibrosis and respectively NASH have iron deposition. We found a positive relation between metal deposition and intensive fibrosis potential, but no association between iron and the other parameters investigated (TGF-beta 1 and CD68 expression).

Liver fibrogenesis represents the common response of the liver to toxic, infectious or metabolic agents. Both clarification of the molecular mechanisms underlying pathological fibrosis and the development of effective therapy are of clinical importance. A central role in fibrogenesis has been assigned to the transforming growth factor-b1 (TGF-b1), which is a particularly well-studied cytokine and widely regarded as a pro-fibrogenic agent in liver injury, particularly after chronic liver injury [10,11]. High levels of TGF-b1 are often found in fibrotic tissues. The cytokine has been implicated as a mediator of fibrosis in many liver diseases [12].

Also, in many liver diseases, iron deposits are found either in hepatocytes, macrophages/sinusoidal cells, or both. However, it's important to mention that excess iron deposits in liver macrophages may affect their immunomodulatory and inflammatory activity, cytokine biology, defense against pathogen and viral infection, immunosurveillance, or response to immunomodulatory drugs [13]. It is known that excessive iron accumulation in the liver causes hepatocellular injury, but the mechanism of tissue injury remains unclear

Some authors believe that iron may be the substrate for oxidative stress and could be responsible for the second hit in patients with NASH [15,16]. In steatotic livers, the saturation of β-oxidation by excess free fatty acids will ultimately lead to the generation of hydrogen peroxide, which in turn can be converted to highly reactive hydroxyl radicals in the presence of free iron via a Fenton reaction [14]. Indeed, there is strong evidence, from both *in vitro* and *in vivo* studies, that iron overload enhances oxidative stress [17-19]. Consistent with several previous findings, the present data showing that serum iron, transferrin saturation, and ferritin levels and the grade of hepatic iron staining (TIS) are significantly higher in NASH compared with simple steatosis also suggest that iron overload may be responsible for the second hit and pathogenesis of NASH. Quantitative analysis revealed that hepatocytic 8-oxodG levels were significantly correlated with these iron-related markers in NASH, strongly indicating that the increase in iron stored in the body is specifically related to increased hepatocytic oxidatively generated damage to DNA in NASH patients.

As iron accumulates, individual hepatocytes will eventually accumulate lethal levels of iron and undergo sideronecrosis. The locally released iron is then taken up into macrophages; however, hepatocellular iron will continue to dominate. Macrophage iron is linked to progressive fibrosis [16]. Precirrhotic portal and periportal fibrosis takes on a 'holly leaf' configuration. When cirrhosis is present, it is bland in nature with fine fibrous tissue septa surrounding regenerative nodules.

Iron deposition has been discussed in numerous studies. Sorrentino et al. found that the amount of iron deposit was significantly higher in HCC-NASH patients than in HCC-free NASH controls [20]. In contrast, Bacon et al. documented elevated hepatic iron concentration in NASH [21]. Other authors state that significant iron accumulation is not seen in most patients with NASH [22,23].

In this study, we have shown an association between high expression of CD68 positive macrophages, TGF-beta 1 and iron deposition on the one hand, and the development of fibrosis in NASH patients. As a result, we conclude that these factors could be a some of the factors that predict development of liver fibrosis and progression of chronic liver disease such as NASH.

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