Liver visfatin expression in morbidly obese patients with nonalcoholic fatty liver disease undergoing bariatric surgery

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Background: Visfatin has been identified as a new adipokine with proinflammatory and immunomodulating properties. It seems to interfere with immune and fibrogenic process in nonalcoholic fatty liver disease (NAFLD). The aim was to assess visfatin expression in the liver tissue and its association with biochemical parameters and morphological features in NAFLD patients.
Material and methods: The study included 40 severely obese patients with NAFLD who underwent intraoperative wedge liver biopsy during a bariatric operation. Immunohistochemical assay was carried out with the use of a visfatin mice monoclonal antibody.
Results: Visfatin expression in the liver was observed in all patients. The expression was significantly higher in patients with fibrosis ($p = 0.036$) and was positively correlated with the fibrosis stage ($r = 0.52$, $p = 0.03$). There was no difference between patient with nonalcoholic steatohepatitis (NASH) and simple steatosis ($p = 0.54$). Inflammatory activity and NAS (NAFLD Activity Score) score were not associated with visfatin expression. There was a tendency of more evident visfatin liver expression in morbidly obese patients with diabetes mellitus.
Conclusion: Our study showed a positive association between visfatin and the fibrosis stage in NAFLD. This observation suggests a potential role of this adipokine in the pathogenesis and progression of NAFLD. Visfatin expression does not seem to be associated with liver steatosis and inflammation.

Key words: visfatin, adipokine, liver, nonalcoholic fatty liver disease, fibrosis, steatosis, obesity.

Introduction

In developed countries nonalcoholic fatty liver disease (NAFLD) has become one of the leading causes of chronic liver disease [1]. Nonalcoholic fatty liver disease presents a wide spectrum of liver injury, ranging from simple steatosis, steatohepatitis (NASH) to cirrhosis, which can further progress to end-stage liver failure [2-4]. In a general population of the Western world the prevalence of NAFLD is greater than 30%, reaching 75-95% in morbidly obese patients. The prevalence of NASH in this subpopulation is 20-54% [3-8].

Since storage of lipids in hepatocytes render the liver more vulnerable to inflammatory and fibrotic reactions, great efforts are made to unravel the pathogenesis of steatosis and to identify the factors promoting development of NASH and its progression to advanced fibrosis [9, 10]. Well – known endocrine and proinflammatory potential of adipose tissue has orientated research interest toward a rapidly expanding group of biochemical mediators named adipocytokines. There is growing evidence that white adipose tissue-derived adipocytokines contribute to development and progression of both liver steatosis and steatohepatitis [11-15].

Visfatin is a recently identified adipocytokine with multipotential activities, secreted not only by adipocytes but also by peripheral, bone marrow and hepatic lymphocytes [16, 17]. On the one hand, visfatin exerts insulin-like activity, showing a direct cardioprotective effect in myocardial infarction [16, 18]. On the other hand, visfatin activates human leukocytes to produce cytokines and adhesion molecules, and contributes to promotion of angiogenesis [19-21]. As this peptide shows proinflammatory and immunomodulating properties, it was suggested to be one of the mediators connecting obesity with inflammation [22]. In NAFLD patients visfatin serum levels were similar to those in obese controls without NAFLD and significantly higher than in non-obese subjects [23]. Moreover, visfatin serum levels in patients with simple steatosis were significantly higher than in patients with NASH and obese controls without liver disease. These observations indicate that visfatin levels increase in obesity and may have a hepatoprotective effect in NAFLD [23].

Percutaneous liver biopsy in morbidly obese patients is very difficult. Very often, intraoperative wedge liver biopsy is the only method allowing the assessment of liver tissue in the morbidly obese.

The role of visfatin as an immunomodulator and mediator of hepatic fibroproliferative processes in NAFLD remains unclear. We hypothesized that visfatin interferes with inflammatory and fibrogenic processes in NAFLD. It is obvious that serum concentrations of adipokines may not reflect their expression on the hepatic level. Therefore, we investigated visfatin expression in the liver and related the expression level to hepatic histological and biochemical features in morbidly obese patients.

Material and methods

The study included 40 severely obese patients with NAFLD who underwent intraoperative wedge liver biopsy during a bariatric operation – biliopancreatic diversion. Mean age was 42.2 ±9.1 years (range 28-50). The duration of severe obesity ranged from 12 to 22 years (mean 14.6 ±5.7). BMI ranged from 42 to 71 kg/m^2 (mean 52.4 ±9.6). Patients with a history of excessive alcohol consumption (more than 20 g/day), drug abuse, autoimmunological or infectious hepatitis, thyroid diseases and HIV infection were excluded. Laboratory data were measured routinely, using standard methods. The upper limit of alanine aminotransferase (ALT) activity was set at 38 UI/L and aspartate aminotransferase (AST) at 40 UI/L. Noninvasive markers of liver tissue alterations were calculated as originally described – AAR = AST/ ALT; APRI = [(AST/ULN)/platelet count (× 10^9)] × 100. A blood sample was withdrawn from all subjects at the beginning of the study, after they had fasted in the morning. The samples were centrifuged and serum was frozen in –70°C until assay.

The liver biopsy samples were stained with heamatoxylin and eosin, azan method for collagen fibres, and examined by two independent experienced pathologists. All liver specimens included more than six portal tracts. Histopathological evaluation was carried out according to Kleiner's scoring scale [24] (Table I). Additionally, a portal inflammatory activity grade was estimated according to Scheuer's scale [25].

The study was approved by the Ethical Committee of the Medical University of Silesia in Katowice and conformed to the ethical guidelines of the Declaration of Helsinki. Informed consent was obtained for the whole study series.

Immunohistochemistry

Immunohistochemical assay was carried out with the use of visfatin (Alexis Biochemical, San Diego, USA) mice monoclonal antibody in 1 : 500 dilution.

Tissue sections, 2 µm thick, were mounted on adhesive slides and incubated at 58°C and then deparaffinized in xylene and re-hydrated in alcohol solutions of decreasing concentrations. After being rinsed in distilled water the sections were stained using immunohistochemical methods.

In order to retrieve the antigen, preparations were placed in TRC solution, catalogue No. S 1699 (Dako, Glostrup, Denmark) in the water bath at 97°C for 30 min. After cooling and rinsing, the endogenous peroxidase was blocked with 3% hydrogen peroxide. Then, sections were incubated for 1 hour with visfatin at room temperature. The remaining immunohistochemical reactions were performed using ABS Universal Kit, cat. No PK 6200 (Vector, Burlingame, USA). The reaction was visualized using Chromogen DAB (Novocastra, Newcastle, Great Britain). Sections were rinsed between consecutive steps with TBS/Tween, cat. No. BUF 028 (Serotec). Sections were counterstained with hematoxyllin, dehydrated, cleared in xylene and coversliped with DPX.

Visfatin expression was assessed with densitometry in bioptate microphotography under 200 magni-

Table I. Kleiner's scoring system

Steatosis – cells involved	
Grade 0	<5%
Grade 1	5-33%
Grade 2	33-66%
Grade 3	>66%
Lobular inflammation – number of foci per × 200 field	
Grade 0	None
Grade 1	<2
Grade 2	2-4
Grade 3	>4
Hepatocyte ballooning – cells involved	
Grade 0	None
Grade 1	Few
Grade 2	Many/prominent ballooning
NAFLD Activity Score (NAS)* – diagnosis of NASH	
≥5	Probable or definitive NASH
3-4	Uncertain
≤2	Not NASH
Fibrosis – staging	
Stage 0	None
Stage 1	Perisinusoidal or periportal
1A	Mild, zone 3, perisinusoidal
1B	Moderate, zone 3, perisinusoidal
1C	Portal/periportal only
Stage 2	Perisinusoidal and portal/periportal
Stage 3	Bridging
Stage 4	Cirrhosis

** sum of points for intensity of steatosis, lobular inflammation and hepatocellular ballooning*

fication using Image-Pro Plus v.3.0 software (Media Cybernetics, Silver Spring, USA). The three largest measured fields were used for analysis.

Statistical analysis

The values were expressed as the mean and standard deviation (± SD). The Shapiro-Wilk test was used to evaluate the distribution. Because of non-gaussian distribution, nonparametric methods were used. Differences in studied variables between groups were tested using U Mann-Whitney and ANOVA rang Kruskal-Wallis tests for independent groups. Correlations were analyzed with the Spearman rank correlation coefficient. $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using STATISTICA 7.0.

Results

The characteristic of the patients studied

Clinical and demographic data of the investigated patients are summarized in Table II.

Table II. Demographic and clinical data in morbidly obese patients with NAFLD

PARAMETR	MEAN ± SD	NORMAL VALUES
sex (M/F)	16/18	–
age (years)	42.2 ±9.1	–
weight (kg)	152.1 ±32.5	–
BMI (kg/m^2)	52.4 ±9.6	–
fasting glucose (mmol/l)	5.3 ±1.7	3.9-5.6
total cholesterol (mmol/l)	6.2 ±1.5	up to 5.2
triglycerides (mmol/l)	2.7 ±1.1	up to 2.0
ALT (IU/l)	39.4 ±20.1	up to 38
AST (IU/l)	33.1 ±19.0	up to 40
alkaline phosphatase (IU/l)	89.8 ±48.7	30-220
total bilirubin (μmol/l)	15.0 ±6.6	4-18
creatinine (μmol/l)	79.5 ±14.2	62-132
WBC (G/l)	7.1 ±1.6	4-10
AAR	0.90 ±0.41	–
APRI	0.57 ±0.50	–

BMI – body mass index, ALT – alanine aminotransferase, AST – aspartate aminotransferase Laboratory biomarkers of advanced fibrosis:
AAR = AST/ALT, APRI = {(AST/ULN})/platelet count (× 10^9)} × 100
WBC – white blood cells

Table III. Results of histopathological examination in morbidly obese patients

HISTOPATHOLOGICAL FEATURES	PATIENTS N	%
steatosis G 0/1/2/3	40 3/12/12/13	100
portal inflammation grades 1/2-4	9 9/0	22
lobular inflammation grades 1/2/3	17 10/5/2	42
ballooning degeneration grades 1/2	15 14/1	38
fibrosis stage 1A/1B/1C/2/3/4	33 3/1/13/10/2/4	83
Mallory bodies	0	0
NASH	24	60
definitive/uncertain	9/15	

N – number of patients; NASH – nonalcoholic steatohepatitis

Histopathological examination of liver tissue samples

The results of histopathological examination are shown in Table III.

Immunohistochemical analysis of visfatin expression

Visfatin expression in the liver parenchyma was observed in all analyzed patients. The mean value of liver tissue visfatin expression was 1.00 ±0.66. Visfatin expression was significantly higher among the patients with fibrosis compared to those without, 1.09 ±0.65 vs. 0.36 ±0.03; respectively; p = 0.036. Comparison of patients with stage 1 fibrosis – F1 (including stage 1A, 1B, 1C) to those with bridging fibrosis/cirrhosis (F3-F4) showed more pronounced visfatin expression among these patients with more advanced fibrosis but the difference did not reach statistical significance – 0.88 ±0.66 vs. 1.23 ±0.68; p = 0.24. In the group of studied patients, statistically significant positive correlation between the fibrosis stage and visfatin expression in the liver tissue was observed (r = 0.52, p = 0.03).

On the other hand, visfatin tissue expression was not associated with either the grade of lobular and portal inflammation or the grade of steatosis (respectively: r = 0.01, p = 0.95; r = 0.16, p = 0.51; r = –0.35, p = 0.34). We did not find statistically significant difference between patients with NASH and those with simple steatosis (1.11 ±0.71 vs. 0.62 ±0.19, p = 0.54). Also, NAS score was not related to visfatin expression (r = –0.26, p = 0.31). There were no differences in visfatin expression index value between patients with NAS 0-2 vs. 3-4 vs. 5-8 (1.05 ±0.56 vs. 0.76 ±0.60 vs. 1.06 ±0.80, p = 0.58, respectively).

The distribution of visfatin expression in lobules was uneven in patients with less advanced fibrosis – stage 1 and 2. The expression was mostly concentrated in and nearby portal tracts. The expression was very weak in the central zone of lobules. In the case of bridging fibrosis the differences in localization of visfatin expression within lobules were not so evident. In the case of cirrhosis, distribution of visfatin expression was diffused.

Visfatin expression in the liver was negatively associated with a patient's body weight (r = –0.69, p = 0.05) but there was no relationship with their BMI (r = –0.39, p = 0.33).

There was a tendency of more evident visfatin liver expression in morbidly obese patients with diabetes mellitus than in non-diabetic subjects (1.72 ±0.60 vs. 0.90 ±0.50, p = 0.044).

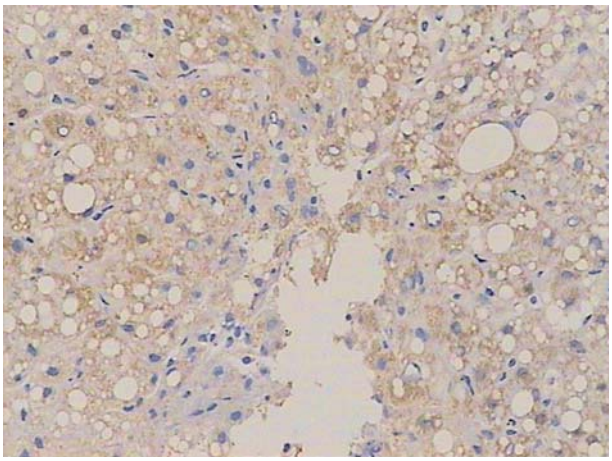

Fig. 1. Liver visfatin expression in patients with periportal fibrosis

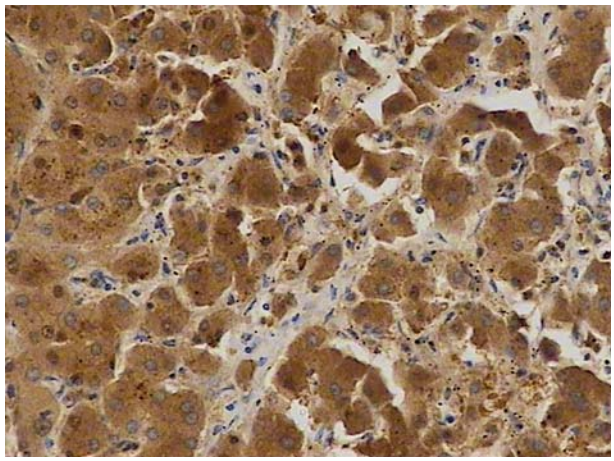

Fig. 2. Liver visfatin expression in patients with cirrhosis

Discussion

Recent data indicate that obesity and type 2 diabetes are predictors of liver fibrosis among patients with chronic liver diseases including NAFLD [6, 12-15, 26]. Accumulating evidence makes it possible to elucidate the cellular and molecular mechanisms linking obesity and insulin resistance with liver fibrosis [13, 26]. The role of visfatin in liver fibrosis remains largely unknown, therefore we decided to investigate for the first time hepatic tissue expression of visfatin in morbidly obese patients with NAFLD.

We found that the liver expression of visfatin in patients with NAFLD was more pronounced in patients with marked fibrosis in comparison to these with no fibrosis. Moreover, liver visfatin expression correlated positively with the stage of fibrosis, being the highest in patients with bridging fibrosis and cirrhosis. The distribution of visfatin expression in lobules was uneven in patients with less advanced fibrosis – stage 1 and 2, mostly localized in and nearby portal tracts where fibrous tissue was observed. When fibrous tissue was more abundant – septal fibrosis or cirrhosis, visfatin expression became diffused. Jarrar *et al*. [23] did not observe any correlation between serum visfatin levels and the fibrosis stage in NAFLD patients. Although Aller *et al*. [27] found that liver fibrosis increased with each unit of fat mass, they were unable to identify visfatin as an agent which influences the fibrosis stage.

Abundance of extracellular matrix is related to specific and complex interplay between fibrogenic tissue metalloproteinases inhibitors (TIMPs) and fibrolytic metalloproteinases (MMPs). In cultured endothelial cells the visfatin was reported to inhibit TIMP 1/2 and induce MMP 2/9 in a dose dependent manner [20]. Moreover, synthesis of MMPs was positively regulated by VEGF, which is up-regulated by visfatin. These observations suggest that visfatin may play an antifibrogenic role in the liver.

Hyperglycemia and insulin resistance/diabetes type 2 are major risk factors of NASH and fibrosis progression [14, 15]. Some data indicate that visfatin lowers glucose serum levels and decreases insulin resistance [16]. Serum visfatin was found to be higher in patients with diabetes than in healthy controls [28]. Our study seems to confirm this finding as visfatin expression in the liver tissue of diabetic patients was higher than in patients with normal glucose tolerance. However, the difference was on a threshold of a statistical significance, probably due to a small number of patients with diabetes.

Accumulation of visceral adipose tissues was recognized as the important risk factor for development of NAFLD, progression to NASH and advanced fibrosis [6, 12, 14]. There are conflicting results regarding relationship between visfatin and obesity. In three recently published studies a significant correlation between the serum visfatin levels and BMI was evidenced [29-31]. Moreover, Fukuhara *et al*. [16] reported a strong positive relationship between plasma visfatin concentration and the amount of visceral fat, while only a weak correlation with thickness of subcutaneous fat was found. Similarly, Filippatos *et al*. [30] observed that the levels of circulating visfatin were higher in patients with metabolic syndrome than in age-matched healthy controls. By contrast, Jarrar *et al*. [23] did not observe any correlation between serum visfatin levels and BMI and the fibrosis stage in NAFLD patients. Pagano *et al*. [32] reported an even negative correlation between obesity and visfatin levels. In our study, liver expression of visfatin was not correlated with BMI, but we observed a tendency for lower visfatin expression in patients with higher BMI and body weight. This observation indicates that adipose tissue as an endocrine organ may influence visfatin liver expression. Therefore, the obtained results in morbidly obese patients should be compared with those in obese and overweight patients.

Recent data show that the main sources of visfatin in adipose tissue are both the adipocytes and macrophages [33]. In patients with severe obesity there is a pathological accumulation of macrophages in adipose tissue [33]. Adipocytes and macrophages produce various proinflammatory cytokines, mainly interleukin (IL)-6 and tumor necrosis factor α (TNF-α). Kralisch *et al*. [34] demonstrated that IL-6 inhibits expression of visfatin in cultured fat cells. This process might, at least partially, explain our observation of negative association between body weight and visfatin expression in liver tissue. In such visfatin expression in the liver is dependent on the amount of fatty tissue and may differ between patients with various types of obesity.

Increased production of visfatin in several acute and chronic inflammatory diseases suggests its role in inflammation [19, 21]. Raised serum TNF-α is an innate feature of obesity and insulin resistance [23, 35]. It is known that visfatin induces TNF-α synthesis in human peripheral blood mononuclear cells and the liver in mice [19]. Moreover, visfatin induces expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) [36]. Aller *et al*. [27] showed that activity of hepatic portal inflammation was associated with an increase of the serum visfatin level in patients with NAFLD. By contrast, Jarrar *et al*. [23] reported that serum visfatin levels were significantly higher in patients with simple steatosis compared to those with NASH. In our study liver visfatin expression showed no relationship with either lobular or portal inflammation or NAFLD Activity Score

(NAS). We also found no correlation of the visfatin expression level with the grade of hepatocyte steatosis. In effect, visfatin expression did not distinguish simple steatosis from NASH.

In conclusion, it has been shown for the first time that hepatic visfatin expression is increased in morbidly obese patients with NAFLD and marked liver fibrosis. Its expression was positively associated with stage of fibrosis. This observation suggests a potential role of this adipokine in the pathogenesis and progression of fibrosis in patients with NAFLD. Visfatin expression does not seem to be associated with pathogenesis of liver steatosis and inflammation. Liver visfatin expression should be assessed in patients with different types of obesity. Intraoperative liver biopsy is very helpful tool for the assessment of new agents responsible for metabolic and pathological processes in the liver in morbidly obese.

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