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RAPID COMMUNICATION

Expression of p53, Bax and Bcl-2 proteins in hepatocytes in non-alcoholic fatty liver disease

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Abstract

AIM: To analyze the protein expression essential for apoptosis in liver steatosis.

METHODS: The expression of proapoptotic proteins p53, Bax, and antiapoptotic Bcl-2 in hepatocytes with steatosis (SH) and without steatosis (NSH) was evaluated in 84 patients at various stages of non-alcoholic fatty liver disease (NAFLD).

RESULTS: Immunohistochemical staining of liver tissue showed the activation of p53 protein in SH and NSH with increased liver steatosis, diminished Bcl-2 and slightly decreased Bax protein. Positive correlation was found between the stage of liver steatosis with p53 expression in SH (r = 0.54, P < 0.01) and NSH (r = 0.49, P < 0.01). The antiapoptotic protein Bcl-2 was diminished together with the advancement of liver steatosis, especially in non-steatosed hepatocytes (r =0.43, P < 001).

CONCLUSION: Apoptosis is one of the most important mechanisms leading to hepatocyte elimination in NAFLD. The intensification of inflammation in NAFLD induces proapoptotic protein p53 with the inhibition of antiapoptotic Bcl-2.

Key words: Apoptosis; Non-alcoholic liver disease; p53; Bcl-2; Bax; Immunohistochemistry

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INTRODUCTION

Liver steatosis results from triglyceride accumulation in hepatocytes in the course of many diseases[1]. The failure of energetic processes in hepatocytes leads to progressive lipid increase in cytoplasm[2]. The most common cause of liver steatosis is alcohol abuse (alcoholic liver fatty disease-ALFD)[3]. The disease accompanies hyperalimentation, obesity, metabolic syndromes, and hyperlipidemia[4,5]. The phenomenon can be the result of side effects of some contraceptive, NSAID, antimetabolic and other drugs[1,3]. Non-alcoholic fatty liver disease (NAFLD) is diagnosed by excluding alcohol abuse and other liver diseases and confirmed by histological changes in the liver and increased aminotransferase activity.

A variety of pathological conditions associated with NAFLD could explain the pathogenesis of the disorder. There is a hypothesis concerning multifactorial conditioning of the disease. Different mechanisms are assumed to lead to NAFLD, including imbalanced fatty acid supply, hyperglycemia, upset hormonal balance between hormones responsible for anabolic and catabolic activities in the portal circulation, and endotoxemia in starvation[2,3]. Oxidative stress that damages lipid peroxidation and mitochondria occurs in chronic liver injury (metabolic disturbances, iron deposition, high fatty acid concentrations). Fatty acid cytotoxic activity is suggested to influence cell surival. Long term accumulation of lipids may lead to hepatocyte necrosis or apoptosis[6]. The mechanisms underlying cell death have not been explained yet. The significant influence of inflammatory and immunological factors, cytokines, and chemokines is probable[7]. The stimulation of mechanisms underlying apoptosis, with the contribution of p53 in hepatocytes has been observed in mice in laboratory conditions[6,8,9].

The aim of our study was to evaluate the expression of proapoptotic p53 and Bax and antiapoptotic Bcl-2 in hepatocytes of patients with liver steatosis. The expression of these proteins, depending on disease advancement and enhancement of steatosis was analyzed in the study.

MATERIALS AND METHODS

Patients

Examinations were performed in 84 patients with liver steatosis (35 women and 49 men, median age 43 years, range 27-62 years). Steatosis was due to obesity, glucose intolerance and chronic use of hepatotoxic drugs. Patients

infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) were excluded from the study. Liver steatosis was confirmed by ultrasonography and biopsy. Liver dysfunction was confirmed by increased aminotransferase values that persisted for longer than 6 mo. The Brunt classification was used to perform the histological analysis of steatosis advancement[10].

Methods

Right lobule liver oligobiopsies were conducted in all patients. Liver biopsies were performed using the Hepafix System 1.6 (Braun, Melsungen, Germany) before treatment. Liver tissues obtained from biopsies were placed in 4% formaldehyde buffered solution for 24 h, then fixed and paraffin-embedded. Five-μm thick sections were routinely stained with hematoxylin and eosin as well as picric acid.

Bcl-2: Tissue sections were put into FSG 120 (FSG120-T/T Controlled Antygen Retrieval, Milestone, Italy) at 110℃ after paraffin was removed and hydration performed in citric buffer (pH 6.0). The pressure was adjusted to 2 bar for 10 min to obtain antigen retrieval. After cooling, the samples were placed in 3% hydrogen peroxide solution for 5 min, then washed in distilled water and placed in fresh buffered Tris for 5 min. The histological preparation was covered with the primary antibody Bcl-2 at 1:40 dilution (anti-human Bcl-2 coprotein, DakoCytomation, Denmark), incubated at room temperature for 30 min and placed in a fresh Tris bath for 5 min. Then, the secondary antibody was added (biotinylated link universal from the commercial kit LSAB: DakoCytomation, Denmark), incubated at room temperature for 30 min before washing in fresh Tris for 5 min. Another secondary antibody was added (streptavidin-HRP from LSAB kit) for 30 min and the section was placed in fresh Tris for 5 min. The preparation was covered with DAB stain (3, 3-diaminobenzidine tetrahydrochloride, DAB Chromogen, DakoCytomation, Denmark) for 10 min and then bathed in distilled water.

p53: The samples were deprived of paraffin, hydrated and placed in a microwave at 600V for 20 min. The procedures described above using anti p53 antibodies in dilution of 1:50 (anti-human p53 protein, DakoCytomation, Denmark) were repeated.

Bax: Preparations were prepared as above. Bax protein was detected using the commercial kit CSA (CSA, DakoCytomation, Denmark) according to the manufacturer's recommendations. The primary Bax antibody was used at dilution 1:1000 (anti-human Bax protein, Dako, Denmark). Then, the preparation was processed.

Using paraffin at a high melting point (65℃) and antigen retrieval in the microwave, we could not detect Bcl-2 and Bax proteins in hepatocytes. On the other hand, using low-melting point paraffin (55℃) and a FSG 120 machine for routine pressurized high temperature antigen retrieval, positive immunohistochemical reactions for Bcl-2 and Bax in hepatocytes were obtained.

The positive reaction showed as brown color of the cytoplasm for Bcl-2 and Bax, and the nuclei for p53. We calculated hepatocyte percentage (the amount of cells with positive reaction in ratio to 100 cells) containing those proteins in 5 fields of vision (× 400) of one slide under a light microscope. Expression of Bcl-2, p53 and Bax was analyzed in steatotic (micro- and macro-droplet steatosis) and non-steatotic hepatocytes. Ethical approval for research was obtained from the Ethics Committee of Medical University.

Table 1 Clinical characteristics of patients with liver steatosis

Clinical characteristic	Grade of steatosis (% of steatosis hepatocytes)		
	< 33% (group Ⅰ)	33%-66% (group Ⅱ)	> 66% (group Ⅲ)
n	24	35	25
BMI	28 kg/m^2	30 kg/m^2	32 kg/m^2
Cholesterol (mmol/L)	5.50 ± 0.75	5.99 ± 1.29	6.80 ± 2.30
Trigliceryde (mmol/L)	2.60 ± 1.27	2.78 ± 1.44	3.16 ± 1.11
Bilirubina (μmol/L)	11.97 ± 5.13[b,d]	17.1 ± 6.84	27.36 ± 20.52
ALT (μkat/L)	0.84 ± 0.35[b,d]	1.70 ± 1.12	1.67 ± 0.38
AST (μkat/L)	0.60 ± 0.33[b,d]	1.19 ± 0.50	1.87 ± 1.80
ALP (μkat/L)	1.12 ± 0.27[b,d]	1.72 ± 0.45	2.10 ± 0.92
GGT (μkat/L)	0.75 ± 0.33[b,d]	2.61 ± 3.42[d]	5.06 ± 4.34
Total protein (g/L)	7.4 ± 04[d]	7.0 ± 0.5[d]	6.4 ± 0.5
Albumin (g/L)	4.5 ± 1.2[d]	4.0 ± 0.2[d]	3.4 ± 0.7

[b]$P < 0.01$ *vs* group Ⅱ, [d]$P < 0.01$ *vs* group Ⅲ (non-parametrical Student's-*t* test).

Statistical analysis

The results were presented as the mean cell number (p53, Bcl-2 or Bax positive hepatocytes) ± SD. The statistical analysis was carried out using non-parametrical Student's-*t* test and Spearman's correlation test.

RESULTS

Histological analysis of liver biopsies showed mixed micro- and macro-droplet steatosis of hepatocytes occurring mainly in the 2nd and 3rd zones of the lobules. Inflammation and fibrosis were not present in group Ⅰ (hepatocyte steatosis < 33%), but were present in group Ⅱ. Moderate periportal and intestitial fibrosis, small necrotic focuses of hepatocytes without necrosis and single lymphocyte clusters were noted in group Ⅲ (steatohepatitis). Malformation in portal spaces was not observed. Table 1 presents the characteristics of the examined groups. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (γ-GT) were increased, total protein and albumin concentrations decreased significantly together with the stage of liver steatosis. There was no significant relationship between the activity of aminotransferase (ALT, AST) and the expression of p53, Bax, Bcl-2 in hepatocytes with SH and HNS.

Bax

Bax expression was significantly higher in steatosed hepatocytes with SH (68% ± 23%) than in non-steatosed hepatocytes (NSH) (59% ± 21%, $P < 0.03$) in the patients with NAFLD (Table 2). The highest Bax expression was observed in SH in group Ⅰ with slight liver steatosis. The intensification of inflammation and fibrosis led to an

Table 2 Percentage of hepatocytes containing pro- and antiapoptotic proteins depending on the stage of liver steatosis (mean ± SD, %)

	Total	Group Ⅰ	Group Ⅱ	Group Ⅲ
Bax NSH[1]	59 ± 21	66 ± 15	52 ± 27	57 ± 19
Bax SH[2]	68 ± 23	71 ± 21	63 ± 28	67 ± 18
p53 NSH[1]	24 ± 12	18 ± 14[d]	21 ± 9[d]	35 ± 9
p53 SH[2]	22 ± 17	14 ± 18[b,d]	20 ± 13[d]	33 ± 14
Bcl2 NSH[1]	55 ± 26	77 ± 21[b,d]	46 ± 20	49 ± 27
Bcl2 SH[2]	44 ± 27	52 ± 37	42 ± 20	41 ± 24

[1]Non-steatosed hepatocytes; [2]Steatosed hepatocytes. [b]$P < 0.01$ *vs* group Ⅱ, [d]$P < 0.01$ *vs* group Ⅲ (non-parametrical Student's-*t* test).

initially insignificant decrease in Bax expression (mainly in NSH). The group of patients with steatohepatitis showed an increased Bax expression mainly in SH. We did not observe any relation between the stage of liver steatosis and the expression of Bax in hepatocytes (Table 3). The expression of p53 in SH ($r = 0.32$, $P < 0.01$) and NSH ($r = 0.33$, $P < 0.01$) was elevated together with the increase in Bax expression. The relationship between Bax expression in NSH and p53 expression in SH and NSH was weak. Together with the increase in alkaline phosphatase in steatohepatitis, Bax expression was enhanced in SH and NSH ($r = 0.57$, $P < 0.01$ and $r = 0.59$, $P < 0.01$ respectively). Gamma-glutamyltransferase elevation was also accompanied by the increase in Bax expression in SH ($r = 0.38$, $P < 0.01$) and NSH ($r = 0.33$, $P < 0.01$).

p53

The expression of p53 was approximately 10% lower in steatosed hepatocytes than in hepatocytes without steatosis (difference was not statistically significant) (Table 2). In the patients with liver steatosis without inflammation (group Ⅰ), p53 expression in SH and NSH was significantly lower than in the patients with steatohepatitis (group Ⅲ). There was a positive correlation between p53 expression and the stage of liver steatosis ($r = 0.49$, $P < 0.01$ in NSH; $r = 0.54$, $P < 0.01$ in SH) (Table 3). The level of albumin was negatively correlated with p53 expression in SH ($r = -0.44$, $P < 0.01$) and NSH ($r = -0.69$, $P < 0.01$). The concentrations of ALP and GGT were positively correlated with positive p53 in SH ($r = 0.36$, $P < 0.01$ and $r = 0.32$, $P < 0.01$, respectively) and NSH ($r = 0.25$, $P < 0.03$; $r = 0.34$, $P < 0.01$, respectively).

Bcl-2

Bcl-2 expression diminished in steatosed and non-steatosed hepatocytes in accordance with the stage of liver steatosis. As far as liver steatosis was concerned, there was a statistically significant defference between positive SH, NSH and Bcl-2 (44% ± 27%, 55% ± 26% respectively, $P < 0.05$) (Table 2). Group I had the highest expression of Bcl-2 in NSH and a lower expression in SH (77% ± 21%, 52% ± 35%, respectively). On the other hand, advanced stage of liver steatosis had a statistically significant decrease in Bcl-2 expression in SH and NSH. A negative correlation was observed between Bcl-2 in NSH and the stage of liver steatosis ($r = -0.43$, $P < 0.01$) (Table 3). However, such a relationship was not observed between the expression of Bcl-2 in NSH and the stage of the disease. The elevation of Bcl-2 expression in NSH increased Bcl-2 expression in SH ($r = 0.46$, $P < 0.01$), Bax expression in NSH ($r = 0.53$, $P < 0.01$) and SH ($r = 0.57$, $P < 0.01$). We did not observe any relationship between the percentage of positive Bcl-2 in NSH and the expression of p53 in SH and NSH. However, there was a correlation between Bax-2 expression in SH and p53 expression in SH ($r = 0.42$, $P < 0.01$) and NSH ($r = 0.36$, $P < 0.01$). No correlation was observed between Bcl-2 expression, alkaline phosphatase and γ-GT activities.

Table 3 Correlation of biochemical degree of liver damage and expression of pro- and anti-apoptotic proteins in hepatocytes with the stage of liver steatosis

	r
ALT	0.6[b]
AST	0.37[b]
ALP	0.55[b]
GGT	0.53[b]
Total protein	-0.54[b]
Albumines	-0.34[b]
Bax NSH[1]	NS[3]
Bax SH[2]	NS[3]
p53 NSH[1]	0.49[b]
p53 SH[2]	0.54[b]
Bcl2 NSH[1]	-0.43[b]
Bcl2 SH[2]	NS[3]

[1]Non-steatosed hepatocytes; [2]Steatosed hepatocytes; [3]Non-statistical correlation. [b]$P < 0.01$ *vs* the stage of liver steatosis (non-parametrical Spearman test).

DISCUSSION

The study revealed that the stage of liver steatosis increased the expression of proapoptotic proteins, mainly p53, in hepatocytes with and without steatosis. Antagonistic protein Bcl-2 was diminished together with the advancement of liver steatosis, especially in normal hepatocytes. Proapoptotic protein Bax seemed to play a minor role in the process of steatosis. Its expression was slightly decreased when steatosis was intensified. There was an apparent difference between hepatocytes with steatosis containing more Bax protein and hepatocytes without steatosis. The pathogenesis of NAFLD is not entirely clear. Triglycerides, localized in the cytoplasm of hepatocytes, are the main component of lipids. Accumulation of lipids can result from insulin resistance, damaged disposal of triglycerides from the cells, β-oxidation damage in mitochondria, or very low density lipoproteins[11]. It has been suggested that free fatty acids (FFA) can play a crucial role in steatosis intensification and necrotic-inflammatory processes. Damage of lysosome integrity, which results in catepsin B and TNF-alpha release, is due to the effect of lipotoxicity. The degree of lysosome instability correlates with NAFLD activity[12].

Cellular free fatty acids show cytotoxic effects, such as

elevation of cytochrome P450 activity[13]. Steatosed hepatocytes are more sensitive to endotoxin, which can result from intracellular IL-10 mRNA reduction and IFN-γ mRNA concentration elevation[13]. It is assumed that chronic oxidative stress in NAFLD influences steatosis progression[14]. Oxidative stress is generated by free fatty acids, pro-inflammatory cytokines, TNF alpha is activated by cytochrome P450 2E1. The disturbances of lipid peroxidation in hepatocytes, which can lead to their extensive accumulation, is the consequence of oxidative stress. Seki *et al*[15] stated that intracellular increase in markers of lipid peroxidase and oxidative DNA damage correlate with the stage of necroinflammatory changes of steatosis in patients with NASH. Chronic oxidative stress induces overexpression of cytochrome P450 2E1 (CYP2E1) in hepatocytes, causing their damage. The mechanism underlying cell death with the participation of CYP2E1 has not been explained yet. Chronic overexpression of p4502E1 increases epidermal growth factor/c-Raf signaling in hepatocytes. Studies conducted by Schattenberga *et al*[16] have proved that hepatocytes exposed to chronic oxidative stress have different sensitivity to noxious stimuli. Cellular resistance to proapoptotic factors has observed in hepatocytes with overexpression of p4502E1 after sensitized by polyunsaturated fatty acids.

Incubation of pancreatic beta-cells with FFA results in a significant increase in cell death due to apoptosis[17]. The cells have a significant drop in Bcl-2 mRNA expression with no change in Bax mRNA expression[17]. Cell death induced by FFA can be blocked by caspase, serine protease, and ceramide synthesis inhibition, suggesting that FFA is the cause of pancreatic beta-cell reduction and leads to peripheral insulin resistance, which plays a role in obesity and liver steatosis[18]. It was observed that FFA administration results in proapoptotic p53 increase and anti-proliferative effect in Caco-2 cells. There is also a simultaneous drop in the level of apoptosis suppressor protein Bcl-2 expression[19].

TNF alpha and CYP2E1, which are more significantly expressed in patients with chronic hepatitis C and steatosis than in those without steatosis, have a role in liver steatosis progression[20]. Ji *et al*[21] suggested that incubation of HepG2 cells with palmitic acid could induce cell death via mitochondria-mediated apoptosis. They also stated that Bax expression is significantly increased but Bcl-2 expression is moderately decreased[21], indicating that liver steatosis influences expression of pro- and anti-apoptotic proteins. Apoptosis is one of the most important mechanisms underlying hepatocyte elimination in non-alcoholic steatohepatitis. The intensification of inflammatory changes in NAFLD induces proapoptotic protein (p53) with the inhibition of antiapoptotic protein (Bcl-2). However, the explanation of mutual relationships between apoptosis and liver steatosis needs further investigation.

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