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Expression of bcl-2 protein in chronic hepatitis C: Effect of interferon alpha 2b with ribavirin therapy

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Abstract

AIM: Mechanisms responsible for persistence of HCV infection and liver damage in chronic hepatitis C are not clear. Apoptosis is an important form of host immune response against viral infections. Anti-apoptotic protein bcl-2 expression on liver tissue as well as the influence of interferon alpha 2b (IFN α 2b) and ribavirin (RBV) were analyzed in patients with chronic hepatitis C.

METHODS: In 30 patients with chronic hepatitis C (responders - R and non-responders - NR) treated with IFN α 2b+RBV, protein bcl-2 was determined in hepatocytes and in liver associated lymphocytes before and after the treatment.

RESULTS: The treatment diminished bcl-2 protein accumulation in liver cells in patients with hepatitis C ($P < 0.05$). Before and after the therapy, we detected bcl-2 protein in R in 87 \pm 15% and 83 \pm 20% of hepatocytes and in 28 \pm 18% and 26 \pm 10% of liver-associated lymphocytes, respectively. In NR, the values before treatment decreased from 94 \pm 32% to 88 \pm 21% of hepatocytes and 39 \pm 29% to 28 \pm 12% of lymphocytes with bcl-2 expression. There was no statistical correlation between bcl-2 expression on liver tissue with inflammatory activity, fibrosis and biochemical parameters before and after the treatment.

CONCLUSION: IFN α 2b+RBV treatment, by bcl-2 protein expression decrease, enables apoptosis of hepatocytes and associated liver lymphocytes, which in turn eliminate hepatitis C viruses.

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Key words: Bcl-2; Chronic hepatitis C; Interferon alpha; Ribavirin; Hepatitis C virus

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INTRODUCTION

Hepatitis C virus is a major frequent agent of chronic liver inflammation. Many aspects play an important role in pathogenesis of HCV infection, e.g., viral genotype, virus-host interactions, local and systemic immune reactions and expression of viral proteins. Hepatitis C virus has cytopathic and viropathic features and induces T lymphocyte cytotoxicity by causing their selective accumulation in the liver. Hepatitis C virus has structural and non-structural proteins, which play a role in replication and production of cellular promoters^[1,2]. Several viral proteins interfere with cellular functions, which are involved in the immune response, cell proliferation and apoptosis in the liver^[3-5].

Apoptosis is an important mechanism limiting viral replication in infected cells. Many viruses produce a variety of mechanisms to neutralize IFN secretion and block genes participating in apoptosis^[6]. Hepatitis C virus has the features of both induction and inhibition of hepatocyte apoptosis. Apoptosis induction upon HCV infection may contribute to liver inflammation, while inhibition of apoptosis may result in HCV persistence and oncogenesis. Modulation of apoptosis by HCV core protein is possible by death receptors, such as Fas, TNF, and lymphotoxin B. The HCV NS3 and NS5A proteins have anti-apoptotic effects^[7,8]. Apoptosis is a result of proapoptotic factor activation (bax, FADD Fas-associated death domain-containing protein, TNFRF1, Fas/CD95) and anti-apoptotic inhibition (bcl-2). The activation of caspase-8 (determined as proapoptotic - b.i.d.) influences the release of cytochrome C from mitochondria and secondary activation of cytoplasmic caspase 9 and 3. Bcl-2 regulates apoptosis through the influence on mitochondrial membrane channel proteins and that decides the amount of released cytochrome C to the cytoplasm^[9-11]. In hepatitis C, apoptosis of parenchymal (hepatocytes) and non-parenchymal cells occurs. The increase of apoptosis of circulating activated T lymphocytes in hepatitis C was observed^[12]. Lack of correlation between caspase activity and the stage of inflammatory changes in the liver is probably caused by this phenomenon. Viral infections are capable of limiting or blocking cell apoptosis in which they reproduce themselves. Core protein among HCV proteins has a crucial role in protecting the cells from apoptosis mediated by anti-Fas and TNF-alpha.

Cytotoxic T lymphocytes induce apoptosis of cells infected with viruses. There is a strict relation between infiltrating lymphocytes and hepatocyte apoptosis. It is suggested that apoptosis is mediated by the host immune system^[8]. Infected hepatocytes show increased Fas expression. In liver inflammatory areas, the increase in Fas ligand expression occurs, which correlates with the stage of hepatitis C^[13]. This is a consequence of T lymphocyte induction after the contact with viral antigen on presented hepatocytes^[14]. Besides Fas-FasL pathway, there is another way through the release of granzyme B/perforin induction of apoptosis in infected cells^[15].

Interferon alpha synthesized by most cell types is a cytokine of multiple properties, e.g., antiviral, immunomodulatory, and antiproliferative properties^[16]. Detailed mechanisms of IFN activity remain elusive. It is assumed that antiviral effect of IFN is possible by induction of apoptosis of infected hepatocytes. The role of IFN in apoptosis and antiviral host defense lies in inducing a number of cellular genes. Hepatitis C virus NS5 and E2 proteins inhibit antiviral activity of IFN by inhibition of IFN signaling pathways and antiviral protein production, e.g., threonine protein kinase^[17].

It seems that hepatocyte apoptosis plays a crucial role in hepatitis C pathogenesis and antiviral activity of interferon alpha may be the effect of apoptosis induction. Therefore, we examined the correlation between liver cell damage and bcl-2 expression on the liver in patients with chronic hepatitis C before and after the treatment with IFN α 2b and RBV.

MATERIALS AND METHODS

Patients

Examinations were conducted in 30 patients, aged 38 ± 13.6 years (14 women and 16 men), with chronic hepatitis C. Chronic hepatitis C lasting for 2.4 ± 1.7 years was confirmed by the presence of anti-HCV antibodies, HCV-RNA in blood serum with elevated alanine aminotransferase in all patients. Patients with liver cirrhosis were excluded from the study. HCV-RNA in sera and biochemical parameters were examined at the end of the treatment and after 6 mo. Patients were divided into responders (R) and non-responders (NR) according to their sustained response to a course of IFN α 2b (3 MU thrice weekly for 48 wk, Rebetrin, Schering-Plough Corporation, USA) with ribavirin (RBV, 1.2 g/d/48 wk, Rebetrin, Schering-Plough Corporation) treatment. Blind liver biopsies were performed using the Hepafix system (Braun, Melsungen, Germany) before the treatment and 6 mo after the therapy. Histopathological inflammatory activity (grading, scales 0-4) and fibrosis (staging, scores 0-4) were evaluated in accordance with the classification of chronic viral hepatitis according to Scheuer classification^[18]. Ethical approval for research was obtained from Local Ethics committee in Medical University.

Methods

Liver tissues obtained from biopsy were placed in 4% formalin buffered solution (24 h), after that they were fixed and paraffin embedded. Five-micrometers of thick sections were routinely stained with hematoxylin-eosin and immunostained by using monoclonal antibodies (isotope IgG1, kappa, clone

DO-7, Dako, Denmark). Primary antibodies were used by the dilution according to the producer's instructions (1:50). A LSAB+HRP kit (Dako, Denmark) with DAB (Dako, Denmark) and chromogen was used as a detective set. The positive reaction was expressed by red-brown color of the cytoplasm for bcl-2. Under a microscope we calculated hepatocytes and lymphocyte percentage (the amount of cells with positive reaction in ratio to 100 cells) containing bcl-2 protein in five fields of vision ($\times 400$) of one slide. Intensity of accumulation of bcl-2 protein in cellular cytoplasm was also evaluated. Intensive immunohistochemical reaction of specific antibodies to bcl-2 protein was determined as grade 1, moderate reaction as grade 2 with high granular intensity of bcl-2 protein accumulation.

Statistical analysis

Results were presented as mean \pm SD. Statistical analysis was performed using Student's *t*-test for pairs, χ^2 test, and Spearman's test.

RESULTS

The clinical characteristics of patients are presented in Table 1. After the treatment, we observed improved inflammatory activity and fibrosis in liver tissue. In four patients we noted progressive liver damage IFN α 2b+RBV therapy diminished accumulation of bcl-2 proteins in all patients with hepatitis C (Table 2). Before the treatment, bcl-2 protein was detected in $92 \pm 12\%$ of hepatocytes and in $37 \pm 26\%$ of liver-associated lymphocytes in all patients. In responders, bcl-2 expression on hepatocytes was $87 \pm 15\%$ and $28 \pm 18\%$, respectively. In patients with successful therapy we observed the lowest percentage of hepatocytes and lymphocytes containing bcl-2 protein before and after the treatment. There were no statistically significant differences in bcl-2 expression on the liver between responders and non-responders.

Together with inflammatory activity (portal, periportal) and liver fibrosis, we noted increased percentages of hepatocytes and liver associated lymphocytes containing bcl-2 protein. However, we did not observe any statistical correlation between bcl-2 expression and histological changes in the liver in patients who revealed both worsening and improvement of inflammatory activity and fibrosis. There was no correlation between bcl-2 expression on hepatocytes

Table 1 Clinical characteristics of patients with chronic hepatitis C (mean \pm SD)

Woman/man (n)	14/16	
Age (yr)	38.0 ± 13.6	
Duration of HCV infection (yr)	2.4 ± 1.7	
	Before treatment	After treatment
ALT/AST (U/L)	$80 \pm 61/78 \pm 46$	$53 \pm 56/65 \pm 41$
ALP/GGT (U/L)	$89 \pm 22/62 \pm 41$	$79 \pm 21/55 \pm 26$
Albumin/gamma globulin (g/dL)	$3.7 \pm 0.4/1.2 \pm 0.3$	$3.9 \pm 0.9/1.3 \pm 0.5$
HCV-RNA positive (n)	30	20
Inflammatory activity (grading), score ¹		
Periportal: 1/2/3/4 (n)	6/8/16/0	10/4/16/0
Intralobular: 1/2/3/4 (n)	18/12/0/0	16/14/0/0
Fibrosis (staging), score: 1/2/3/4 (n)	20/8/2/0	16/12/2/0

¹According to Scheuer classification (1991).

Table 2 Expression of bcl-2 protein on hepatocytes and lymphocytes in liver tissue before and after treatment with IFN α 2b and RBV (mean \pm SD)

Chronic hepatitis C, total, <i>n</i> = 30	Before treatment	After treatment	<i>P</i>
Hepatocytes (%)	92 \pm 12	87 \pm 11	<0.05
Liver-associated lymphocytes (%)	37 \pm 26	27 \pm 21	NS
Responders, <i>n</i> = 10			
Hepatocytes (%)	87 \pm 15	83 \pm 20	NS
Liver-associated lymphocytes	28 \pm 18	26 \pm 10	NS
Non-responders, <i>n</i> = 20			
Hepatocytes (%)	94 \pm 32	88 \pm 21	<0.05
Liver-associated lymphocytes (%)	39 \pm 29	28 \pm 12	NS

and lymphocytes, and activity of alanine aminotransferase before and after the treatment. Analyzing the intensity of bcl-2 accumulation in cells, we observed a high granular accumulation of bcl-2 proteins in hepatocytes and in liver associated lymphocytes in hepatitis C patients (Table 3). The treatment achieved the decrease in bcl-2 positive cell percentage and the density of protein in cells. There was no statistically significant difference between responders and non-responders concerning bcl-2 accumulation before and after the treatment. A statistical correlation between bcl-2 intensity on lymphocytes and liver fibrosis was observed in responders ($r = 0.7$, $P < 0.01$) after IFN α 2b+RBV treatment.

DISCUSSION

The results proved that the initiation of apoptosis in chronic hepatitis C led to HCV elimination. All patients treated with IFN α 2b+RBV showed a significant reduction in bcl-2 accumulation in hepatocytes and lymphocytes. It proves that IFN α 2b+RBV could affect bcl-2 concentrations in the liver, and stimulate apoptosis of hepatocytes and lymphocytes. Whether it is a direct effect of antiviral drugs on bcl-2 or a result of HCV elimination is unknown. Immunohistopathological study revealed that apoptosis was much inhibited in non-responders than in responders before the treatment. In NR, bcl-2 expression was also decreased, although to a less extent than in responders, under the influence of IFN α 2b+RBV therapy.

HCV proteins could modulate apoptosis of hepatocytes by indirect or direct mechanisms^[3,19]. Mechanisms of immune-mediated apoptosis in the liver are the most probable. Degree of liver apoptosis correlates positively with histological inflammatory activity and with level of infiltrating CD8 T cells. Apoptosis is regulated by products of various gene expressing bcl-2 inhibitors (bcl-2, bcl-xl, mcl-10) and apoptosis promoters (so called "killers" bax, bak, bok, bad, which contain domains of BH1, BH2, BH3)^[9]. The bcl-2 proto-oncogene is localized in the inner mitochondrial membrane of cells and blocks programmed cell death. Over-expression of *bcl-2* gene could lead to apoptosis inhibition and enable cell survival. Sensitivity of cells to factors inducing apoptosis may also be regulated by pro- and anti-apoptotic protein ratio.

Calabrese *et al*^[8], showed that apoptotic index of hepatocytes in liver tissue ranged from 0.01% to 0.54%, and presented a positive correlation between histological

Table 3 Intensity of bcl-2 protein expression on hepatocytes and liver lymphocytes in patients with chronic hepatitis C during IFN α 2b and RBV therapy

Intensity of accumulation	Bcl-2 in hepatocytes (%)		Bcl-2 in liver lymphocytes (%)	
	Before	After	Before	After
High granular (<i>n</i>)	21	9	15	13
Moderate (<i>n</i>)	9	21	15	17
χ^2 test	$P > 0.1$		$P > 0.5$	

activity and value of CD8 cells in liver tissue. However, the relation between apoptosis intensity and aminotransferase level as well as HCV viremia and genotype was not observed. Apoptosis could also occur without elevated transaminases. There was no correlation between biochemical activity and liver cell injury. Our study did not show any statistical significance between bcl-2 protein expression and histological activity and liver fibrosis.

Rubbia-Brandt *et al*^[20], revealed that 50% of patients with hepatitis C had apoptotic hepatocytes, 52% Fas receptor expression, and 30% tumor necrosis factor receptors (TNFR). Fas- and TNFR-positive hepatocytes did not correlate with the value of intrahepatic CD8+ T cells, the grading and staging of liver diseases, or the serum or liver HCV-RNA levels^[20]. Many factors, such as HCV NS proteins, could decide bcl-2 expression. Hepatitis C virus core protein could bind to a domain of lymphotoxin B receptor (LTBR), which is a member of the tumor necrosis factor receptor family^[21]. It was reported that LTB receptor was involved in lymph node development^[22]. Thus, it should be assumed that HCV core protein plays a potential role in induction of host immune mechanisms. It seems that hepatitis C virus may cause both necrosis and apoptosis of hepatocytes.

HCV-specific T cells migrate to the liver and recognize viral antigens and affect the expression of Fas ligand, which enables transmission of apoptotic death signals with induction of apoptosis. Fas antigen is a cell surface protein that mediates apoptosis in hepatitis C. Hayashi and Mita^[23] observed a high prevalence of Fas expression mainly in the cytoplasm of hepatocytes and in infiltrating lymphocytes in liver tissues with severe inflammation. Inflammatory cells could secrete cytokines (mainly TNF α), inducing apoptosis through surface receptors^[24]. Ray *et al*^[25], suggested that HCV core protein inhibited TNF α -mediated apoptosis and might enhance HCV replication with persistent infection. Pharmacological inhibition of apoptosis may favor chronic HCV infection and even intensify its replication. Persistent infection of HCV might be a result of suppression of Fas- and TNF- α -mediated cell death and inhibition of apoptosis of liver cells^[20,26].

HCV infection could induce caspase activation, which correlates closely with the inflammatory response. HCV proteins could inhibit the activation of caspases-9 and 3/7 and release of cytochrome C from mitochondria. Bcl-2 protein could inhibit apoptosis of infected cells in a caspase-dependent manner. Caspase activation and apoptosis did not correlate with biochemical activity (aminotransferase levels), viral load, and genotype^[4]. It is possible that apoptosis occurs not only in hepatocytes, but also in other cells.

Furthermore, the release of transaminases was lower from apoptotic cells than from necrotic cells. It could explain the progression of liver diseases in asymptomatic patients with HCV infection and normal values of transaminases^[7]. It is believed that caspase activity monitoring may be an index of liver injury and efficacy of antiviral therapy (IFN alpha, RBV).

It seems that apoptosis is a principal process leading to HCV elimination. But activation of this process is not clear. Antiviral treatment is a potent signal to apoptosis activation but future research is required.

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