Specific ssDNA concentration in liver tissue as an index of apoptosis in hepatitis C virus-infected patients

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INTRODUCTION
Activity of apoptosis during hepatitis C virus (HCV) infection is a result of inhibitory and stimulatory effects of viral proteins. Apoptosis of HCV-infected hepatocytes influences the elimination of viruses. Insufficient activity of this process can lead to persistent liver inflammation[1]. On the contrary, excessive activity of apoptosis causes uncontrolled damage to liver cells. Moreover, there is evidence that apoptosis influences fibrosis progression.

This study was to evaluate the activity of apoptosis in liver tissue and explore its possible association with hepatic necroinflammation and fibrosis activity as well as serum HCV viral load.

MATERIALS AND METHODS
Patients
The study included 50 chronic hepatitis C patients (20 women and 30 men, aged 18-66 years). Their HCV infection did not exceed 5 years. Inclusion criteria were the following: no focal changes in ultrasonography investigation of liver, absence of drugs or alcohol abuse, autoimmune disease, HIV and hepatitis B co-infection as well as neoplasmatic and other serious diseases, which might alter apoptosis activity. Informed consent was obtained from each patient and the Bioethics Committee at the Medical University of Bialystok approved the study protocol.

The presence of anti-HCV antibodies in serum was detected by MEIA (Abbott, USA). Test was based on recombinant core proteins for HCr43, structural proteins for c200 (NS3 and NS4) and unstructured proteins for c100-3 (NS4) and NS5.

HCV-RNA quantification methods
Blood samples were obtained from 47 patients with known chronic hepatitis C. All blood samples were collected to Vacutainer tubes with no additives and centrifuged within 2 h of collection. The serum was aliquoted and kept at -80 °C until further use.

RNA was extracted from 280 μL of each serum sample using Qiagen (Hilden, Germany) QIAamp viral RNA mini kit according to the manufacturer’s protocol and dissolved in 50 μL of RNase-free water. This resulted in nearly sixfold concentration of the original output material. RNA was stored at -80 °C until further testing. Each isolation was carried out in duplicate.

HCV-RNA quantification was performed by two-step real-time quantitative RT-PCR method using the TaqMan technology[2]. First, 3.5 μL of total RNA was subjected to reverse transcription in 10 μL of reaction mixture using the Applied Biosystems (Applera Corporation, USA) TaqMan

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reverse transcription reagents and random hexamers (d(N))6. 
RNA was previously denatured at 70°C for 10 min. The reaction 
mixture was prepared as follows: 1× reverse transcription 
buffer, 5.5 mmol/L MgCl2, 0.5 mmol/L of each dNTPs, 
2.5 mmol/L of primer (d(N))6, 0.4 U of RNase inhibitor per 
µL and 1.25 U of MultiScribe transcriptase per µL. The 
reaction was carried out at 48°C for 30 min preceded by 
the incubation at 25°C for 10 min. Incubation at 95°C for 
5 min terminated the reaction. The resulting cDNA was 
used as a template for PCR amplification.

cDNA amplification was performed by the Applied Biosystems (Applera Corporation, USA) ABI Prism 7 900HT 
sequence detection system in TaqMan universal PCR master 
mix, which is optimized for TaqMan reactions and contains 
Applied Biosystems (Applera Corporation, USA) AmpliTaq 
gold DNA polymerase, AmpErase UNG, dNTPs with UTP, 
passive reference ROX and optimized buffer components. 
For each PCR reaction, 4 µL of cDNA was added to 16 µL 
of PCR mix containing 450 nmoL each of HCV-specific 
forward and reverse primers and 300 nmoL of HCV- 
specific FAM-MGB probe. The primers and probe were 
designed and manufactured by Applied Biosystems (Applera 
Corporation, USA) and optimized for HCV genotype RNA 
detection. Thermal cycling conditions were designed as 
follows: inactivation of possible contaminating amplicons 
by AmpErase UNG at 50°C for 2 min, initial CDNA 
denaturation at 95°C for 10 min, followed by 40 cycles at 
95°C for 15 s and at 60°C for 60 s. All amplification reactions 
were carried out in triplicate.

Fluorescent measurements were recorded during the 
annealing step of each cycle. At the end of each PCR run, 
data were automatically analyzed by the system to generate 
the amplification plots and the threshold cycle (Ct) for each 
sample was calculated. Sample RNA concentrations were 
then automatically calculated by interpolation of the experi-
mentally determined standard curve.

An external standard curve was generated with each run 
by amplification of 10-fold serial dilutions of RNA isolated 
from the viral quality control serum (Peli Spy, Sanguin), 
containing 38 000 geq/mL HCV. RNA isolation and cDNA 
synthesis were carried out as described for unknown samples. 
All amplification reactions were carried out in duplicate. The 
standard curve was created automatically by the ABI Prism 
7 900HT detection system by plotting the Ct values against 
each standard dilution of known concentration.

Precautions were undertaken to minimize the risk of 
PCR contamination during analysis.

Liver morphology
Liver tissue was obtained from the right lobe of the liver (with 
1.6-1.8 mm needles from Hapafix packs by Braun, Germany). 
A part of the liver tissue was subjected to morphologic 
examination. The morphology of liver tissue was assessed 
 descriptively and scored (necroinflammatory activity and 
 fibrosis) according to Scheuer[3].

Another part of the liver tissue was transferred to 0.9% NaCl 
buffer and then subjected to apoptosis activity measurement.

Apoptosis in liver tissue
The early apoptosis activity in liver tissue was measured by 
ssDNA apoptosis ELISA kit, (Chemicon, Germany) based 
on the mAb to single-stranded DNA (ssDNA). The intensity of 
the reaction between ssDNA and mAb was evaluated. The 
amount of ssDNA was calculated with reference to absorbance 
line of positive and negative control.

The liver tissue was homogenized in 0.9% NaCl buffer. 
The concentrations of ssDNA and total proteins in the 
homogenized liver tissue were analyzed in duplicate. The 
homogenates of liver tissue were transferred to wells 24 h 
 prior to the succeeding steps of the procedure to allow cell 
attachment. Then formamide was added to denature DNA 
in apoptotic cells, but not in necrotic cells or in cells with 
DNA damage in the absence of apoptosis. This process 
enabled specific receptors of apoptotic ssDNA binding to 
mAb. Following this reaction, 3-ethylbenzazolene-6-sulfonic 
acid was added to color the products of this reaction[4].

Absorbance was defined at 405 nm. The concentration 
of ssDNA was calculated from a prepared standard curve 
of absorbance (basis of different increasing positive and 
negative control). The results of ssDNA were referred to 
1.0 g of proteins in the liver tissue sample.

Statistical analysis
Statistical analysis was performed by non-parametric Mann-
Whitney U, Wilcoxon rank, and Pearson tests. P<0.05 was 
considered statistically significant.

RESULTS
Diagnostic biopsy was obtained from 47 patients. In three 
of the remaining cases, the liver biopsy sample did not contain 
five portobilary areas, therefore it was impossible to obtain 
reliable histology results.

The activity of apoptosis in liver tissue of the studied 
group was independent of sex and age. ssDNA concentration 
in liver tissue did not correlate with ALT, AST activity, 
prothrombin time, and INR index. Moreover, apoptosis 
was independent of necroinflammatory changes in liver 
tissue (Table 1).

|                  | ALT | AST | Prothrombin time | INR index |
|------------------|-----|-----|------------------|-----------|
| Pearson test     | 0.08| 0.14| -0.01            | 0.03      |

The correlation between fibrosis and apoptosis was observed. 
Concentration of ssDNA was higher (16.65×10-3 µg/g) in 
patients with more intensified fibrosis of liver tissue than 
in those with fibrosis (12.71×10-3 µg/g). However, this 
difference was not statistically significant (P>0.05). (Figures 
1 and 2)

The serum HCV-RNA concentration did not correlate 
with ALT, AST activity, prothrombin time, and INR index. 
Moreover, no correlation between serum HCV-RNA and 
ssDNA concentrations in liver tissue was observed (Table 2).

DISCUSSION
Fas particles are “death domains” and indicators of apoptosis. A and suppressing TNF-α apoptosis by diminishing synthesis of caspase 3, protein kinase can increase the activity of apoptosis. Higher Fas concentration in patients with HCV infection on hepatocytes, leading to degradation of cell DNA[5,6]. The or suppress apoptosis.

HCV core proteins overactivate Fas particles expressed on hepatocytes, leading to degradation of cell DNA[28]. The Fas particles are “death domains” and indicators of apoptosis. Higher Fas concentration in patients with HCV infection can increase the activity of apoptosis.

HCV non-structural proteins (NS5A) are able to suppress apoptosis by diminishing synthesis of caspase 3, protein kinase A and suppressing TNF-α stimulating polymerase[29]. Insufficient activity of apoptosis can influence the persistence of chronic inflammation related to HCV infection[31].

Di Martino et al[5], showed that activity of apoptosis in chronic hepatitis C patients is proportional to HCV-RNA levels. Caronia et al[10], have shown that HCV strongly inhibits apoptosis. In previous investigations, the concentration of ssDNA, as an early indicator of apoptosis was not correlated with HCV viral load, functional state of the liver and necroinflammatory changes in liver tissue samples.

Tsamandas et al[11], evaluated the location and concentration of Bcl-2 and Bax protein, markers of apoptosis activity, in patents with HBV and HCV infection, and found that Bcl-2 and Bax concentrations are enhanced in the regions of intense fibrosis. Takehara et al[12], have shown the linear dependence between the concentration of Bcl-2 family members and activity of fibrosis in the liver. Our results are in accordance with their observations.

Bcl-2 and Bax proteins can mark the late period of apoptosis. Correlation between apoptosis and fibrosis in liver tissue seems unquestionable. However, whether cells with high activity of apoptosis are molecular inductors of fibrosis in HCV-infected patients remains to be solved.

In conclusion, fibrosis of liver tissue is associated with HCV-infection, but apoptosis is not associated with necroinflammatory changes in chronic hepatitis C patients.

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