**RESULTS:** Twenty-four (71%) healthy HBsAg carriers showed mo after treatment.

**METHODS:** Thirty persons with chronic HBV infection treated with lamivudine and patients with chronic HCV infection treated with interferon alpha and ribavirin. Activity of apoptosis was evaluated by serum sFas/sFasL concentration measurement. Moreover dependence between apoptosis and HBV-DNA or HCV-RNA levels was studied.

**RESULTS:** Eighty-six persons were included into study: 34 healthy HBsAg carriers, 33 patients with chronic HBV infection and 19 patients with chronic HCV infection. Serum levels of sFas/sFasL were measured by ELISA assay. HBV-DNA and HCV-RNA were measured by RT-PCR assay. Levels of sFas/sFasL were determined before and 2 and 12 wk after therapy in patients with chronic hepatitis B and C infection. HBV-DNA or HCV-RNA was detected before treatment and 6 mo after treatment.

**RESULTS:** Twenty-four (71%) healthy HBsAg carriers showed HBV-DNA over 10^5/mL, which was comparable to the patients with chronic hepatitis B. Independent from HBV-DNA levels, the concentration of sFas among healthy HBsAg carriers was comparable to healthy persons. Among patients with chronic hepatitis B and C, the concentration of sFas was significantly higher in comparison to healthy HBsAg carriers and healthy persons. In chronic hepatitis B patients the concentration of sFas was decreased during lamivudine treatment. Among chronic hepatitis C patients the concentration of sFas was increased during IFN alpha and ribavirin treatment. sFasL was not detected in control group. Furthermore sFasL occurred more frequently in chronic hepatitis C patients in comparison to chronic hepatitis B patients.

**CONCLUSION:** There are no correlations between apoptosis and HBV-DNA levels. However, there is an association between apoptosis and activity of inflammation in patients with chronic HBV infection. Apoptosis can be increased in patients with chronic hepatitis B by effective treatment which may be a result of apoptosis stimulation by IFN-α.

**INTRODUCTION**

Apoptosis is one of the factors prolonging inflammation in chronic hepatitis B and C. Impaired apoptosis activity or its hyperactivity may induce unfavourable course of HBV and HCV infection. Fas and FasL are proteins that regulate activity of apoptosis. Fas is dominantly localized on liver cells. Activation of Fas is an after-effect of fusion with FasL lymphocyte receptors. These particles are recognized defining coefficient state of stimulation apoptosis. The concentration of Fas/FasL in serum patients with chronic hepatitis B and C can define the success of therapy.

The aim of this study was to define the activity of apoptosis among healthy HBsAg carriers and patients with chronic hepatitis B and C. Apoptosis was defined by use of sFas and sFasL serum concentration measurements. Moreover the apoptosis activity was compared with HBV-DNA or HCV-RNA serum levels. sFas and sFasL levels were monitored during antiviral therapy and afterwards depending on treatment effectiveness.

**MATERIALS AND METHODS**

The study included 86 persons divided into three groups: HBsAg carriers (n = 34, 15 women and 19 men, aged 20–43 years), chronic hepatitis B patients (n = 33, 10 women and 23 men, aged 18–76 years), and chronic hepatitis C patients (n = 19, 6 women and 13 men, aged 18–76 years). Criteria for inclusion of HBsAg carriers group were: HBsAg presence for at least 1 year; ALT, AST activity, concentration of bilirubin, albumin and PT in normal range in one-year period of observation (triple investigation); no changes in USG investigation of liver; absence of drug or alcohol abuse, autoimmune disease, HIV, delta virus or hepatitis C coinfection, and neoplastic and other serious diseases which might alter apoptosis activity. Criteria for inclusion of chronic hepatitis B patients were: HBsAg, HBeAg and HBV-DNA in serum for 6 mo; ALT activity over 80 IU; inflammatory changes in histological evaluation of liver tissue. All chronic hepatitis B patients were treated with nucleic analogue - lamivudine (Zeffix®, GlaxoSmithKline, Great Britain) in a dose of 100 mg/24 h for 12 mo.

Criteria for inclusion of chronic hepatitis C patients were: presence of anti-HCV and HCV-RNA in serum for 6 mo, ALT activity over 80 IU and inflammatory changes in histological evaluation of liver tissue.

Patients with chronic HCV infection were treated with Rebetrion® (IFN-α 2 b and ribavirin, Schering-Plought, USA); IFN-α 2 b in 9 M IU/wk and ribavirin 1 000-1 200 mg/24 h for 12 mo.

The standard concentration of sFas and sFasL in serum was evaluated in 12 healthy persons, 8 women and 4 men, aged 26–46 years. Individuals in control group were HBs and anti-HCV negative and ALT activity in serum was within normal range during 6 mo observation.

**Methods**

HBsAg and HBeAg in serum were detected by MEIA test (ABBOTT, USA).

**Extraction of HBV DNA from sera of patients**

HBV-DNA was extracted from 200 µL of patients’ sera the gene
elute mammalian genomic DNA Miniprep kit (Sigma, USA) for DNA isolation. Part of HBV-DNA was amplified by PCR system with pair of complement primer to conservative part of genome (sense 5'-AGG GGG AGG AGA TTA GTG TAA-3' antisense 5'-AGG AGT GCC AAT CCA CAC-3') in 20µL reaction mixture containing 200µmol/L dNTPs, 0.4µmol/L all primers, 1.5 mmol/L MgCl2, 1.0 U Taq polymerase (Sigma) and 4 µL DNA solution. Forty cycles of amplification were performed (at 96 °C for 30 s, at 57 °C for 60 s and at 72 °C for 60 s). The products of amplification were appointed in 20 g/L agar gel by electrophoresis, and stained by ethidium bromide. Electrophore was visualized in record system and computer analysis UVI-KS400/ Image PC (Syngen Biotech, USA). Part of solution from DNA was kept at temperature -20 °C for further stages of work.

In order to detect HBV-DNA sequences in sera of the patients, the conventional PCR method with primers to conserved pre-S/S region of the genome was used. Thermal cycling was performed using the following conditions: initial incubation at 96 °C for 120 s, and then 40 cycles at 94 °C for 30 s, at 50 °C for 30 s and at 72 °C for 60 s.

HBV-DNA concentration in sera was evaluated by real-time detection PCR based on TaqMan chemistry. Amplification was performed in 25 µL reaction mixture containing 2xTaqMan Universal Master Mix (Applied Biosystems, USA) with uracil glycosylase, 30 pmol of forward primer, 30 pmol of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 µL of isolated DNA. After incubation for 2 min at 50 °C, which enabled uracil N'-glycosylase, 30 µL of forward primer, 30 µL of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 µL of isolated DNA. After incubation for 2 min at 50 °C, which enabled uracil N'-glycosylase to inactivate possible contaminating ampiclons, incubation for 10 min at 95 °C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N'-glycosylase. Next cycles of PCR were moved. Number of copies was counted by interpolation from definite standard curve.

Presence of anti-HCV antibodies in serum was detected by MEIA (ABBOTT, USA). Test consisted of recombined core proteins for HCr43, structural proteins for e 200 (NS3 and NS4) and unstructured proteins for c 100-3 (NS4 and NS5).

**Extraction of HCV-RNA from blood of patients**

HCV-RNA was isolated from 50 µL blood of patients. Parts of viral HCV-RNA were amplified in arrangement of RT nested PCR reaction with two peers nested primers, complementary to conservative part of genome virus (external primers: sense 5'-TCT AGC CAT GGC GTT AGT GTG TT-3', antisense 5'-CATTCCGAA ACC CTA TAG TAT CAG GCC GTG GT-3'; internal primers: sense 5'-GGC GAC ACT CAC CCA CCA TAG AT-3', antisense 5'-GTG CAC GTG CTA CGA GAC CT-3'). Stages of reverse transcription as well as first PCR were executed in one test-glass in 20 µL reaction mixture containing 200 mmol/L dNTPs, 1 mmol/L from each steam external primer, 1.5 mmol/L MgCl2, 0.75 mmol/L MnSO4, 2.0 At Tth of polymerase (EPICENTRE®, USA) as well as 8 µL RNA. After 30 min of incubation at 60 °C for 40 cycles of amplification were executed (at 94 °C for 30 s, 55 °C for 60 s, at 72 °C for 60 s). A 1 µL product of first PCR was amplified with pair of internal primers in 20 µL reaction mixture containing 200 mmol/L dNTPs, 0.4 µmol/L from each primer, 1.5 mmol/L MgCl2, 1.0 U Taq polymerase (Sigma, USA). Afterwards, 40 cycle of amplification were performed (at 94 °C for 30 s, at 55 °C for 60 s, at 72 °C for 60 s). The products of amplification were evaluated in 20 g/L of agar gel by electrophoresis and stained by brom etidyne. The electrophore was visualized in VI-KS400i / Image of PC system (Syngen Biotech, USA).

The minimum detectable dose of HCV was 50 copies per mL of serum.

**Concentration of sFas and sFasL**

The sFas/sFasL concentration in serum was measured twice by quantitative sandwich enzyme immunoassay (ELISA) technique (Bender Med Systems, Austria).

Informed consent was obtained from each patient and the Bioethics Committee at the Medical University of Bia³ystok approved the study protocol.

**Statistical analysis**

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

**RESULTS**

Twenty-four (71%) of 34 healthy HBsAg carriers showed active HBV replication. The HBV-DNA levels exceeding 10⁵/mL were observed in 22 (65%) HBsAg carriers. According to National Institutes of Health, such persons should be considered for antiviral treatment as chronic hepatitis B patients[4,5]. All individuals in chronic hepatitis B group presented HBV-DNA levels exceeding 10⁵/mL.

Independently from HBV-DNA levels, the concentration of sFas among HBsAg carriers and healthy persons was comparable (x = 15.4 ng/mL, x = 18.4 ng/mL, P=0.05). Among patients with chronic HBV or HCV infection, the concentration of sFas was significantly elevated in comparison to healthy persons and healthy HBsAg carriers (x = 29.0 ng/mL, x = 28.0 ng/mL, P<0.05), (Figure 1A). There were no statistical differences between chronic hepatitis B and C patients (Table 1).

### Table 1 Serum sFas concentration and frequency occurrence and concentration of sFasL in serum of patients

|                      | n  | sFas- | patients with sFasL (%) | sFasL- | ng/mL | ng/mL |
|----------------------|----|-------|------------------------|--------|-------|-------|
| Healthy              | 12 | 18.4  | 0                      | 0      | 0     | 0     |
| Total healthy HBsAg carriers | 34 | 15.4  | 56                     | 0.9    | 0.6   | 0.6   |
| Healthy HBsAg carriers | 12 | 13.6  | 58                     | 0.7    | 0.8   | 0.8   |
| HBV-DNA <10⁵/mL or 0 | 22 | 16.3  | 55                     | 1.0    | 1.0   | 1.0   |
| Chronic hepatitis B  | 33 | 32    | 32                     | 0.18   | 0.18  | 0.18  |
| Chronic hepatitis C  | 19 | 28    | 68                     | 1.0    | 1.0   | 1.0   |

sFasL was not detected in control group. This protein was identified more frequently in chronic hepatitis C patients than in chronic hepatitis B patients.

ALT activity reached normal values during the first three months of lamivudine treatment in all chronic hepatitis B patients (114 IU before treatment vs 32 IU after 3 mo of therapy, P=0.001). In the same period the concentration of sFas was decreased in all patients (before treatment x = 29.0 ng/mL, after 14 d of therapy x = 25.0 ng/mL, after 3 mo of therapy x = 22.0 mg/mL). The concentration of ALT was observed 2 wk after therapy in patients with chronic hepatitis C (102 IU before treatment vs 30 IU after 14 d of therapy). All of the chronic hepatitis C patients included in our study had undetectable HCV-RNA after 6 mo of treatment. The highest concentration of sFas was observed in chronic hepatitis C patients with virological response to antiretroviral treatment. The concentration of this protein kept increase during therapy (all HCV infected patients: before treatment x = 28.0 mg/mL, after 14 d x = 31.0 mg/mL, after 3 mo of therapy x = 30.0 mg/mL, patients with HCV infection had good response: before treatment x = 35.0 mg/mL, after 14 d x = 39.0 mg/mL, after 3 mo of therapy x = 39.0 mg/mL, (Figure 1B). The number of patients with sFasL occurrence in serum was the highest before and 3 mo after treatment (83%) among all patients who were effectively treated.
DISCUSSION

Fas and Fasl are “death domain” proteins which regulate the process of apoptosis in patients with chronic inflammatory diseases, such as viral hepatitis[6-9]. The Browicz-Kupffer cells participate in the process. It stimulates synthesis of cytokines regulating biosynthesis of Fas in hepatocytes. Recent studies indicated that it was dependent on the liver or serum sFas concentration and apoptosis activity as well as hepato-cytotoxic processes in viral chronic hepatitis patients[10,11].

In this study we showed that the concentration of sFas in healthy persons and healthy HBsAg carriers was comparable and not dependant on the HBV-DNA levels. It was confirmed trough the lack of correlation between HBV-DNA levels and apoptosis. The concentration of sFas was significantly higher in patients with chronic hepatitis B infection than in healthy HBsAg carriers, independent similar levels of HBV-DNA (>10^5/mL).

Interestingly a large number of healthy HBsAg carriers presented sFasL in serum. The possible explanation of this fact is that HBV might stimulate expression of FasL expression in patients, with HBV-DNA levels and HBsAg carriers with independent similar levels of HBV-DNA (>10^5/mL).

Increased expression of FasL on peripheral blood mononuclear cells might be caused by IFN-α apoptosis stimulation [11-13]. This fact is a probable explanation of the increased number of patients presenting sFasL during antiviral therapy. It may also confirm the beneficial influence of apoptosis in controlling chronic inflammation during HCV infection.

sFas serum concentration in chronic hepatitis B and C patients was comparable before treatment, but after antiviral therapy the concentration of this protein behaved in a different way. These differences underlined the diverse pathogenesis of HCV infection patients compared to healthy persons, as well as a further raise in sFas serum levels during antiviral therapy. Moreover, we observed an association between high activity of apoptosis expressed as serum sFas concentration and sFasL occurrence, and antiviral treatment effectiveness.

Increased expression of FasL on peripheral blood mononuclear cells might be caused by IFN-α apoptosis stimulation[21]. This fact is a probable explanation of the increased number of patients presenting sFasL during antiviral therapy. It may also confirm the beneficial influence of apoptosis in controlling chronic inflammation during HCV infection.

sFas serum concentration in chronic hepatitis B and C patients was proportional to HCV-RNA levels. Ozaslan et al.[20] suggested a positive dependence on chronic hepatitis C intensity and serum sFas concentration.

Our study showed a significant increase of sFas concentration in HCV infection patients compared to healthy persons, as well as a further raise in sFasL serum levels during antiviral therapy. Moreover, we observed an association between high activity of apoptosis expressed as serum sFas concentration and sFasL occurrence, and antiviral treatment effectiveness.

In conclusion, inflammation activity is associated with apoptosis in chronic hepatitis B, and there is no correlation between apoptosis and HBV-DNA concentrations. In HCV infection the effective therapy is accompanied with high activity of apoptosis, which can be a result of IFN-α activity.

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