HBV-DNA and sFas, sFasL concentrations in serum of healthy HBsAg carriers

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Abstract

Purpose: **Increased HBV-DNA concentration is a prognostic factor of disease progression in chronic hepatitis B patients. Moreover, active hepatic inflammation during HBV replication influences apoptosis intensification. The aim of this study was to estimate occurrence of HBV replication among carriers of HBsAg. Furthermore, we analysed the correlation between HBV replication and HBeAg or anti-HBe presence as well as known apoptosis indicators – sFas and sFasL concentration.**

Material and methods: **The study included 34 HBV infected patients, aged 20-43 yrs defined as HBsAg healthy carriers. HBV-DNA was extracted from patients' serum using two different DNA isolation kits: the QIAamp DNA Mini Kit (QIAGEN Ltd, USA) and the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA).**

HBV-DNA concentration in serum was measured by RT-PCR based on TaqMan Universal Master Mix (Applied Biosystems). The detection limit of this system was as few as 10 HBV-DNA copies/mL of serum. HBV-DNA concentration was calculated from a linear standard curve obtained between 10 and 10^8 DNA copies/reaction.

HBeAg and anti-HBe in serum were detected by MEIA method (ABBOTT, Germany). The concentration of sFas and sFasL in serum was – estimated by ELISA method (Bender MedSystems, Austria).

Results: **HBV active replication was detected in 79% HBsAg carriers. The HBV-DNA levels exceeding 10^5 copies/mL were observed in 64% patients. Among HBsAg carriers presenting HBeAg, HBV replication occurred more often and was more intensify than in HBsAg carriers presenting anti-HBe antibodies. The sFasL occurrence in serum of 56% HBsAg carriers shows an active apoptosis, independent from ALT and AST activity within normal ranges.**

Key words: HBsAg carriers, HBV-DNA levels, apoptosis.

Introduction

Healthy HBsAg carriers show correct liver function. Some of those persons eliminate HBV. However, Hou et al. [1] showed, progressing liver inflammation in approximately 30% of chronic HBsAg carriers. Exact prognostic factors defining a direction of an HBV infection evolution in such persons are not known. It seems that high HBV replication may is suggest the developments of chronic liver inflammation.

HBeAg presence in serum of persons with high HBV replication indicates the infection of "wild" type HBV. Among patients that show anti-HBe antibodies existence, replication of HBV is frequent and is mainly caused by mutant HBV infection, namely YMDD [2,3]. The HBsAg carriers with high levels of HBV-DNA are a group of increased risk of chronic liver inflammation development. Antiviral therapy initiation should be considered in such patients.

Patients with active hepatitis B present escalation of apoptosis and cytotoxic reactions. One of the indicators of this process is high Fas and FasL serum concentration. Simultaneous confirmation of a high HBV-DNA replication and the Fas, FasL serum concentration in HBV infected patient should indicate a need of an antiviral therapy consideration [4].

The aim of this study was to define the frequency of HBV replication among HBsAg carriers as well as to investigate the HBV-DNA levels in reference to HBeAg, anti-HBe presence and Fas, FasL serum concentrations.

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Material and methods

The study included 34 HBV infected patients, 15 women (aged 20-43 yrs) and 19 men (aged 21-38 yrs) defined as HBsAg healthy carriers.

Inclusion criteria were:
1. age 18 and over
2. chronic HBV infection evidenced by HBsAg presence for at least 1 year
3. ALT, AST, bilirubin, albumin and prothrombin time within normal range in period of yearly observation (triple investigation)
4. no changes in USG liver examination.

Patients presenting one or more of following criteria were excluded:
1. significant laboratory abnormalities in ALT or AST activity
2. past or present history of drug or alcohol abuse
3. autoimmune disorders (ANA in serum), past or present
4. HIV, delta virus or hepatitis C co-infection
5. patients undergoing dialysis because of chronic renal injury
6. history of malignant diseases.

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

Extraction of HBV-DNA from patient sera

HBV-DNA was extracted from 200 μl of patient sera using two different kits for DNA isolation: the QIAamp DNA Mini Kit (QIAGEN Ltd, USA) and the "Gene Elute Mammalian Genomic DNA Miniprep Kit" (Sigma, USA). In the first method (QIAamp DNA Mini Kit) 200 μl of serum was added to 200 μl lyses solution (AL) supplemented with 5 μg of oligo $(A)_{25}$ carrier DNA and 400 μg of QIAGEN protease. After 10 minutes at 56°C incubation, 230 μl of 96% ethanol was added and the mixture was transferred onto a QIAamp spin column combined attached to a collective tube. The microcolumn was centrifuged for 1 minute at 6000xg in a standard tabletop centrifuge at room temperature. After being washed once with 500 μl of washing buffer (AW1) and once with washing buffer (AW2), DNA was eluted with 50 μl of elution buffer AE (10 mM Tris-HCl, 0.5 mM EDTA-Na_2, pH 8.0). This resulted in obtaining fourfold concentration of the original input material.

In the second method (Genomic DNA Miniprep Kit) 200 μl of serum was added to 200 μl lyses solution with 400 μg of proteinase K. After 10 minutes at 70°C incubation for, 200 μl of 96% ethanol was added and the mixture was transferred onto a spin column attached to a collective tube. The microcolumn was centrifuged for 1 minute at 12000xg in a standard tabletop centrifuge at room temperature. After being washed twice with 500 μl of washing buffer DNA was eluted with 200 μl of elution buffer AE (10 mM Tris-HCl, 0.5 mM EDTA-Na_2, pH 8.0).

Obtained DNA solutions were stored at -20°C until further processing.

All serum samples were processed at the same time with both negative (water) and positive control (HBV-positive serum) samples. To minimize the risk of samples cross-contamination, only one tube of serum was opened at a time during the aliquoting and lyses steps.

HBV-DNA quantification

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

We detected HBV-DNA concentration in sera by real-time detection PCR based on TaqMan chemistry. Amplification was performed in 25 μl reaction mixture containing 2 x TaqMan Universal Master Mix (Applied Biosystems) with uracil N'-glycosylase, 30 pmol of forward primer, 30 pmol of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 μl of isolated DNA. The primers and probe were selected in the pre-S region of the HBV genome and generated a product of 89 bp. After incubation for 2 minutes at 50°C, which enables uracil N'-glycosylase to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N'-glycosylase. The PCR cycling program consisted of 45 two-step cycles of 15 w at 95°C and 60 s at 60°C. Analysis of raw data was done with the Sequence Detector V1.6.3 software (PEBiosystems). Data were collected at the annealing step (60°C) of every cycle, and the threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit, which was set at 0.04 U. The standard curve was calculated automatically by plotting the Ct values against each standard of known concentration. For preparation of the external standards an international reference VQC plasma preparation panel (CLB) containing well-characterized HBV-DNA levels was used. Sample copy numbers were calculated by interpolation of the experimentally determined standard curve. The detection limit of this system was as few as 10 HBV-DNA copies/ml of serum. A linear standard curve was obtained between 10 and 10^8 DNA template copies/reaction.

Serological analyses

HBV infection was diagnosed on the basis of HBsAg, HBeAg and anti-HBe the presence. The HBsAg, HBeAg and anti-HBe were detected by microenzyme immunological method (MEIA, ABBOTT, Germany).

sFas and sFasL

The sFas and sFasL concentration in the serum was measured twice by immunology – enzymatic assay test (ELISA, Bender MedSystems, Austria) [5].

Statistical analyses

Statistical analysis was performed with use of Logistic Regression test and Fisher Exact test by program of SS for Windows. Values of $p<0.05$ were considered to be significant.

Table 1. **The HBV-DNA and sFas, sFasL serum concentration in healthy HBsAg carriers presenting HBeAg or anti-HBe antibodies**

No	sex	HBeAg	anti-HBe	HBV-DNA			HBV-DNA	sFas** pg/ml	SFasL*** pg/ml
				QIAGEN*	NESTED*	SIGMA*	QIAGEN* real-time		
1	♂	-	+	-	++	-	8.4x10^5	18	0.13
2	♀	-	+	+	-	-	1.06x10^5	16.5	0.1
3	♀	+	-	-	-	-	0	13.5	0
4	♀	-	+	+	-	+	2.08x10^5	8	2.57
5	♂	-	+	++	+	-	9.09x10^4	14	11.5
6	♀	-	+	+	-	-	4.1x10^4	8	2.53
7	♂	-	+	++	-	-	3.04x10^5	31	0
8	♂	-	+	++	-	-	9.18x10^5	8.5	0
9	♂	-	+	+++	+++	++	6.27x10^5	19	0
10	♂	-	+	++	+	-	4.43x10^5	7.4	0.13
11	♂	+	-	+	+	-	2.72x10^5	14	0.08
12	♀	-	+	++	++	-	9.82x10^6	7.5	0.08
13	♀	-	+	++	++	++	0	3.5	0.16
14	♂	-	+	-	-	-	1.39x10^5	43	0.83
15	♂	-	+	-	++	-	1x10^4	8	0
16	♀	+	-	+	+	-	1.33x10^5	19	0
17	♀	+	-	+	-	-	0	16	0
18	♂	+	-	+	-	+	2.95x10^5	6.5	0
19	♂	+	-	+++	++	+	2.45x10^5	14	9.89
20	♀	-	+	-	-	-	0	11	0
21	♀	-	+	+	++	-	5.77x10^4	22	0.08
22	♀	-	+	+	-	-	4.15x10^4	12.5	0.12
23	♀	-	+	+	++	+	1.45x10^5	13	0
24	♂	-	+	++	++	+	1.43x10^6	12.5	0
25	♀	-	+	-	-	+	0	17	0.81
26	♂	+	-	++++	++	+++	1.93x10^9	13	0
27	♀	+	-	++	-	-	1.01x10^5	23	0
28	♂	+	-	++++	++	+++	8.03x10^9	27	0.08
29	♂	-	+	++	++	+	9.85x10^5	25.5	0
30	♂	-	+	++	++	+	2.09x10^5	13	4.73
31	♂	-	+	+	++	-	0	31	0
32	♂	+	-	+++	+++	-	5.57x10^6	11.5	2.53
33	♂	-	+	-	-	-	0	7	0.07
34	♀	-	+	+	+	+	1.1x10^5	7.5	0.1

* – DNA isolation method; ** – normal range = 20.3 pg/ml, S.D. = ±4.9; *** – normal range = 0

Results

Our investigations showed that the QIAGEN test is a more sensitive in HBV-DNA detection in comparison to SIGMA test; however, we did not observed statistical differences between used tests.

HBV replication was confirmed in 24 (71%) of 34 HBsAg carriers. 22 (65%) of them presented HBV-DNA level exceeding 10^5 copies/mL. Moreover, 8 (80%) of HBsAg and HBeAg positive carriers showed HBV-DNA replication over 10^5/mL. Among HBsAg carriers presenting anti-HBe antibodies, only 14 (54%) had HBV-DNA concentration exceeding 10^5/mL. We did not observe significant differences level in HBV-DNA between HBsAg carriers in respect to HBeAg and anti-HBe antibodies occurrence. The HBV viral load below 10^4 copies/mL was observed only in HBsAg carriers with anti-HBe antibodies, (5/24 persons =21%).

The difference in sFas serum concentration between HBsAg carriers and control group (15.4 pg/ml vel 18.4 pg/ml; p>0.05) was not significant. Concentration of sFas in serum did not depend on HBeAg or anti-HBe antibodies presence (15.75 pg/ml vel 15.18 pg/ml; p>0.05).

None of individuals in control group expressed sFasL in serum. Conversely sFasL was detected in sera of 19 (56%) HBsAg carriers, which may indicate increased apoptosis activity in these patients.

Discussion

The HBsAg carriers are defined as healthy persons, thus do not require antiviral treatment. Furthermore, according to the National Institutes of Health, patients with serum HBV-DNA levels exceeding 10^5 copies/mL of HBV-DNA in serum should

be considered as a individuals with an active HBV replication and ongoing liver damage, thus should be classified as chronic hepatitis B [6]. Investigations oindicating correlation between histological changes in liver and HBV-DNA serum concentration may confirm this opinion. It is particularly apparent in patients whose HBV viral load in serum exceeds 10^6 copies/mL [7].

Several evidences suggest that advancement of liver inflammation is correlated with HBV viral load [1]. This is coherent with studies showing the relationship between the amount of liver inflammation and HBV-DNA in HBsAg carriers with suppression of immunological response [8]. Langer et al. [9] found active HBV-DNA replication in 49% of HBsAg carriers. Those persons didn't have any symptoms and biochemical markers of liver function were within normal ranges.

For a long-time HBeAg to anti-HBe seroconversion was recognised as a profitable indicator of HBV elimination. Tedder et al. [10] describedd a relationship between HBV-DNA viral load and the presence of HBeAg. On the other hand, Chu et al. [11] and Langer et al. [9], found higher proportion of anti-HBe antibodies in HBsAG carriers with active viral replication than in patients presenting HBeAg. The interesting observation was that in some individuals after HBeAg to anti-HBe seroconversion HBV-DNA concentration decreased [10,12].

Karasawa et al. [13], in 93% HBsAg carriers with anti-HBe antibodies detected HBV mutant "pre-core" existence. Moreover, investigations of Shan's et al. [14], showed a higher rate of HBV mutations among anti-HBe presenting carriers than in patients with HBeAg. It is possible that mutation process contributes to low level of HBV-DNA in persons presenting anti-HBe antibodies.

HBV active replication among HBsAg carriers leads to chronic hepatitis development. Furthermore, long lasting stimulation of hepatocytes to Fas synthesis may induce inflammatory – necrotic changes and fibrosis intensification in the liver [15].

Fas and FasL are proteins that regulate processes of apoptosis in patients with chronic liver injury [16-18]. The Kupffer' cells stimulate the synthesis of cytokines influencing biosynthesis of Fas in hepatocytes. In their study of Xin et al. [19] and Luo et al. [20] confirmed relationship between Fas and FasL serum or liver's tissue concentration and apoptosis activity in patients with chronic liver injury. Cytotoxic reaction and apoptosis run simultaneously in chronic hepatitis B patients and both are controlled by Fas and FasL synthesis [21]. Results of our investigations suggest that sFasL incidence in HBsAg carriers may reflect apoptosis activation. Regardless of correct biochemical markers of the liver function sFasL detection in the serum of HBsAg carriers may demonstrate activation of non-specific immunological processes against HBV infection.

Conclusions

High HBV replication is common within HBsAg carriers. It concerns healthy HBsAg carriers presenting HBeAg as well as anti-HBe antibodies in serum. However, the HBV replication is slightly decreased in carriers with anti-HBe antibodies incidence. The sFasL occurrence in over a half of studied HBsAg carriers may demonstrate activation of non-specific immunological processes against HBV infection.

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