Concentrations of ssDNA in liver tissue and its correlation with sFas and sFasL in serum of patients infected with HBV, HCV, HCV and HIV

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Abstract

Purpose: The concentration of nucleic acids that undergo apoptosis (ssDNA) determines the actual activity of programmed cell death. ssDNA concentrations in liver tissue of patients with chronic HBV, HCV and HCV and HIV infections were assessed. The concentration of this nucleic acid was analyzed in relation to the concentrations of serous apoptosis indicators, sFas and sFasL receptor proteins, the activity of inflammatory processes and fibrosis in liver tissue as well as HBV, HCV and HIV viraemia.

Patients: The study included 153 patients: 48 chronic HBV infected, 86 chronic HCV infected and 19 HCV and HIV infected.

Patients and methods: The concentrations of HBV-DNA, HCV-RNA and HIV-RNA were determined by use of RT-PCR method. CD3+, CD4+ and CD8+ lymphocytes count were detected in HIV infected patients' blood by use of a flow cytometer. The concentration of ssDNA was determined by use of monoclonal antibodies and ELISA tests. The concentrations of sFas and sFasL in serum were determined by use of an immunoenzymatic method (ELISA).

Results: The concentration of ssDNA in liver tissue of both HCV and HBV infected patients was higher in comparison to those co-infected with HCV and HIV ($1\,332\times10^{-6}$ µg/mg, ±664×10-6; vs $1\,508\times10^{-6}$ µg/mg, $\pm810\times10^{-6}$; vs 886×10^{-6} µg/mg, $\pm388\times10^{-6}$; $p<0.004$). No correlation between ssDNA concentration and HBV and HCV viraemia was observed. In patients infected with HCV genotype 3, the concentration of ssDNA was $1\,343\times10^{-6}$ µg/mg, $\pm700\times10^{-6}$, comparable from patients infected with genotype 1, 296×10^{-6} µg/mg, $\pm615\times10^{-6}$. The highest concentration of ssDNA in liver tissue was detected in HBV infected patients with low inflammatory activity ($1\,645\times10^{-6}$ µg/mg, ±987) and low fibrosis ($1\,606\times10^{-6}$ µg/mg, $\pm876\times10^{-6}$). Mild inflammatory changes and low fibrosis were observed in all HCV and HIV infected patients. No correlation between ssDNA concentration in liver tissue and HIV viraemia ($r=0.03$; $p=0.90$), HCV, CD8+ and CD4+ count ($r=-11$; $p=0.66$) was observed. The concentration of ssDNA among HCV and HIV infected patients correlated with the concentration of sFas in serum ($r=0.52$; $p<0.02$).

Conclusions: HCV, HBV and HIV viraemias do not correlate with ssDNA concentration in liver tissue. In patients with HCV and HIV infections, CD4+ and CD3+ counts do not correlate with the concentration of ssDNA in liver tissue. HIV infection seems to inhibit apoptosis processes in liver tissue of HCV and HIV co-infected patients. In the case of HCV and HIV infections, the concentration of sFas in serum correlates with the concentration of ssDNA in liver tissue.

Key words: HCV, HBV, HIV infection, hepatocytes apoptosis.

Introduction

Hypo- or hyperactivity of apoptosis processes can influence the persistence of a chronic inflammatory state in the liver. Most of examinations assessing apoptosis activity in HBV and HCV infected patients are based on the determination of the concentrations of programmed death cell indicators in blood. The concentration and activity of these indicators can be modified by modulators, such as Bcl-2, or indicate simultaneous inflammatory necrotic processes. The determination of the concentration of cellular nucleic acids that undergo apoptosis (ssDNA) unambiguously indicates a programmed cell death process in progress. The aim of the study was to assess the concentration

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of ssDNA in liver tissue of patients with chronic HBV, HCV, HCV and HIV infections. The concentration of the nucleic acid was analyzed in relation to the concentration of serous apoptosis indicators, sFas and sFasL receptor proteins, the activity of inflammatory processes and fibrosis in liver tissue as well as HBV, HCV and HIV viraemia in serum.

Patients and methods

The study included 153 patients: 48 chronic HBV infected (17 women and 31 men aged 19 to 63), 86 chronic HCV infected (32 women and 54 men aged 18 to 66) and 19 HCV and HIV infected (4 women and 15 men aged 19 to 48). All the patients under study had been qualified and waiting for antivirus treatment due to chronic viral hepatitis.

HBV-DNA quantitative assay

HBV-DNA was detected by use of PCR method using a conservative pre-S/S primer. The concentration of HBV-DNA in serum was determined by means of RT-PCR method, using TaqMan chemistry (Applied Biosystems, USA) reagents. Amplifications were performed by use of a 25-µl TaqMan Universal Master Mix. For detection, Sequence Detector V1.6.3 (PEB bisosystems) was used. A standard line for HBV-DNA copies was drawn by means of standard sera. Copies count could range from 10 to 108 in a milliliter.

HCV-RNA quantitative assay

The RNA of HCV virus was amplified in a RT-nested – PCR reaction system with two pairs of nested primers, complementary to the conservative part of viral genome (external primers: sense 5'-TCT AGC CAT GGC GTT AGT ATG AGT GT-3', antysense 5'-CAC TCG CAA GCA CCC TAT CAG GCA GT-3'; internal primers: sense 5'- GGC GAC ACT CCA CCA TAG AT-3', antysense 5'-GTG CAC GGT CTA CGA GAC CT-3', (Sigma, USA). Amplification products were detected by means of electrophoresis in 2.0% agarose gel stained with ethidium bromide. Electropherograms were visualized in a Syngen Biotech UVI-KS400i/Image PC documentation and computer analysis system and quantitative concentration was determined.

HIV-RNA quantitative assay

The infection was diagnosed on the basis of double detection of HIV antibodies in blood by means of an immunoenzymatic method (ELISA, ABBOTT, USA) and Western-blot[1] confirming test (Cambridge Biotech Corporation, USA). HIV virus copies count was determined by means of RT-PCR method using Cobas Amplicor HIS 1.5 (Ultra Sensitive)[2].

Studied lymphocytes count

CD3+, CD4+ and CD8+ lymphocytes counts were determined in HIV infected patients' blood by means of a Becton Dickinson flow cytometer[2].

The concentration of ssDNA in liver tissue

The formamide is an agent that denatures DNA in apoptotic cells, but not in necrotic cells or in the cells with DNA breaks in the absence of apoptosis. In apoptotic cells, formamide denatures DNA to single-stranded DNA (ssDNA) [1].

The concentration of ssDNA was determined by means of Apoptosis ELISA Kit ssDNA test, (CHEMICON, Germany). The tissue obtained during biopsy was examined morphologically and its fragment was placed in 0.9% NaCl buffer solution. The tissue was emulgated and then the concentration of proteins in it was determined. The suspension of emulgated cells was transported to chambers for 24 hours in order to fix hepatocytes to their walls. Formamide, which denatured DNA in cells undergoing apoptosis facilitated the detection of ssDNA. The complexes of ssDNA and monoclonal antibody were stained and absorbance was determined by means of a 405 nm beam spectrometer. The concentrations of ssDNA were assessed by means of a plotted absorbance curve. The obtained results were re-calculated to one gram of liver tissue.

The concentration of sFas and sFasL

The concentrations of sFas and sFasL in serum were performed twice, using an immunoenzymatic method (ELISA, Bender MedSystems, Austria). The sFas and sFasL proteins were bound with monoclonal antibodies and then stained and optical density of sera at the wavelength of 450 nm was measured. The concentrations of sFas and sFasL were determined by comparing optical density to the plotted standard curve of the concentrations of sFas or sFasL.

Morphological assessment of liver

Liver tissue obtained from transcutaneous bioptates of this organ was analyzed morphologically in accordance with Scheuer's classification.

All patients and control group individuals gave their consent to take part in the study. The approval for the study was obtained from the Bioethical Committee of the Medical University of Białystok.

Statistical analyses

Statistical analyses were performed using Mann-Whitney and Spearman tests. Significance level $p<0.05$.

Results

The concentration of ssDNA in liver tissue does not depend on sex and age of patients [TW Lapiński et al. World J Gastroenterol, 2005; 11: 6130-3].

The concentration of ssDNA was higher in liver tissue of HCV infected patients ($1\,332\times10^{-6}$ µg/mg, $\pm664\times10^{-6}$), in comparison to those co-infected with HCV and HIV (886×10^{-6} µg/mg, $\pm388\times10^{-6}$; $r=2.84$; $p<0.004$) and in HBV

[1] The Western-blot test was performed in Laboratory and Experimental Institute of Department of Venerology Medical University of Warsaw, Head: Z. Solibórska, M.D., Ph.D.

[2] The examination was performed in Department of Immunology and Molecular Diagnosis of Regional Infectious Hospital in Warsaw, Head: J. Stańczak, M.D., Ph.D.

Table 1. **Concentration of ssDNA in liver tissue of HBV, HCV and HCV and HIV infected patients in relation to inflammation and fibrosis (Scheuer's classification)**

patients		ssDNA, µg/mg			
		inflammation 0-2	inflammation 3-4	fibrosis 0-1	fibrosis >1
HBV infected	x	1 645*	1 331	1 606**	1 135
	±SD	987	466	876	277
HCV infected	x	1 361*	1 301	1 344**	1 300
	±SD	613	722	662	682
HCV and HIV infected	x	886*	-	886**	-
	±SD	388	-	388	-

*, ** – statistical differences

Table 2. **Differences of studied parameters in HIV and HCV co-infected patients in relation to the number of CD4**

Group of patients	n		ssDNA x 10^{-6} µg/mg	CD8+ µl	HCV copy/mL	HIV copy/mL
CD4+ <410 µl, x=322 µl; ±SD=59	9	x	829	625	3.8 x 2 Log10	3.0 x 3 Log10
		±SD	327	170	1.7 x 1 Log10	6.1 x 3 Log10
CD4+ >410 µl, x=495 µl; ±SD=54	10	x	937	960	3.5 x 3 Log10	6.9 x 3 Log10
		±SD	447	494	2.4 x 1 Log10	8.9 x 3 Log10
p<0.0002			p=0.62	p=0.08	p=0.19	p=0.46

Table 3. **The concentration of ssDNA in liver tissue in relation to viraemia and HCV genotype**

Group of patients	viraemia or genotype	n	ssDNA, x 10^{-6} µg/mg	
			x	±SD
HCV	HCV > 3 log10	54	1356	704
	HCV < 3 log10	32	1209	470
	genotype 1	68	1461	530
	genotype 3	18	1104	448
HCV/HIV	HCV > 2 log10	11	892	339
	HCV < 2 log10	8	878	471
	HIV > 3 log10	8	948	520
	HIV < 3 log10	11	841	275

infected patients (1 508×10^{-6} µg/mg, ±810×10^{-6}) in comparison to those co-infected with HCV and HIV (886×10^{-6} µg/mg; ±388×10^{-6}; r=3.55; p<0.0004). No difference as to the concentration of ssDNA in liver tissue between HBV and HCV infected patients was observed (r=-1.02; p=0.3) – *Fig. 1*.

Among HBV and HCV infected patients, the concentration of ssDNA in liver tissue was higher in persons with higher viraemia but it did not correlate with HBV (r=0.21, p=0.14) and HCV (r=-0.01; p=0.92) viraemias. Among patients infected with HCV genotype 3 the concentration of ssDNA was comparable from infected with genotype 1 (1 343 µg/mg × 10^{-6}, ±700 × 10^{-6}; vs 1 296 10^{-6} µg/mg, ±615 × 10^{-6}). No correlation between the concentration of ssDNA and ALT activity was detected – *Tab. 3*.

The highest concentration of ssDNA in liver tissue was detected in HBV infected patients, with low inflammatory activity (1 645 × 10^{-6}µg/mg, ±987 × 10^{-6}) and low fibrosis (1 606 × 10^{-6} µg/mg, ±876 × 10^{-6}). Mild inflammatory changes and low fibrosis were observed in all HCV and HIV infected patients. HBV and HIV infected patients, in comparison to HBV infected patients with similar histopathological changes, had lower concentration of ssDNA (886 x 10^{-6} µg/mg, ±388 × 10^{-6}; vs 1 645 × 10^{-6} µg/mg; ±987 × 10^{-6}; r=3.13, p<0.002). As regards HCV infection, there were similar correlations of the concentration of ssDNA in relation to HCV and HIV co-infection (1 361 × 10^{-6} µg/mg, ±613 × 10^{-6}; vs 886 × 10^{-6} µg/mg, ±388 × 10^{-6}; r=3.08, p<0.002). When comparing patients with similar fibrosis progression, differences in the concentration of ssDNA were observed between HBV infected patients and those with HCV and HIV (1 606 × 10^{-6} µg/mg vs 886 x 10^{-6} µg/mg; r=3.61; p<0.0003) as well as between HCV infected patients and those

Figure 1. **The concentration of ssDNA in liver tissue of HBV, HCV and HCV and HIV infected patients**

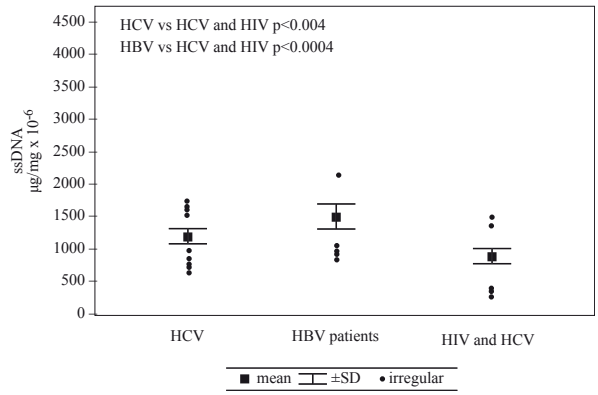


***Figure 2*. The concentration of sFas in serum of HBV, HCV and HCV and HIV infected patients**

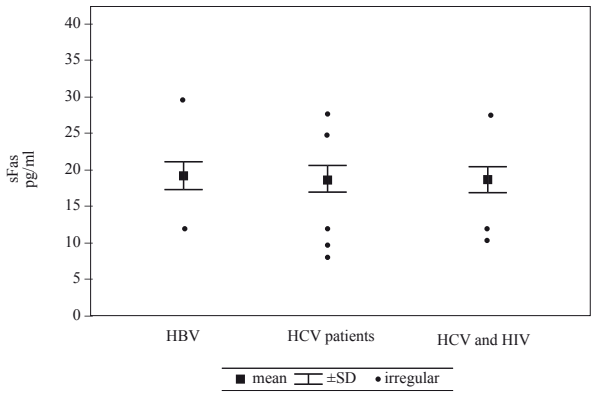
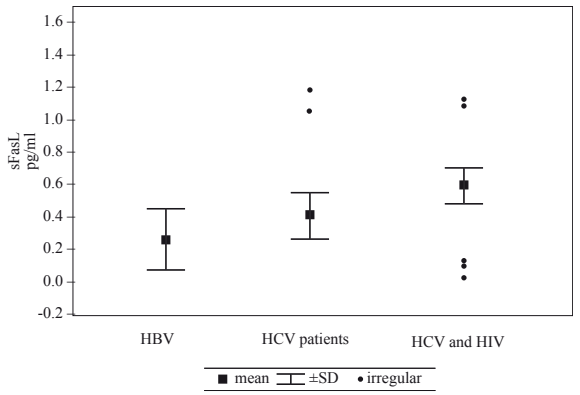


***Figure 3*. The concentration of sFasL in serum of HBV, HCV and HCV and HIV infected patients**



with HCV and HIV (1 344 × 10^{-6} µg/mg vs 886 × 10^{-6} µg/mg; z=2.88; p<0.004) – *Tab. 1*.

No correlation between ssDNA concentration in liver tissue and HIV viraemia (r=0.03; p=0.90), HCV, CD8+ and CD4+ counts (r=-11; p=0.66) was observed. Among HIV and HCV infected patients, the concentration of ssDNA in liver tissue was higher in patients with higher HIV (841 × 10^{-6} µg/mg, ±275 x 10^{-6}; vs 948 × 10^{-6} µg/mg, ±520 x 10^{-6}) and HCV (878 × 10^{-6} µg/mg, ±471 × 10^{-6}; vs 891 x 10-6 µg/mg, ±339 × 10^{-6}) viraemias. These differences, however, were not statistically significant – *Tab. 2*.

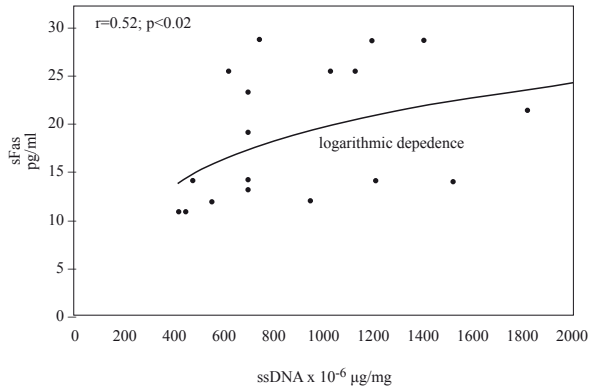
The mean concentrations of sFas in sera of patients infected with HBV, HCV, HCV and HIV were comparable – *Fig. 2*.

The concentration of sFasL was the highest among HCV and HIV infected patients. The differences were not statistically significant – *Fig. 3*.

Only among HCV and HIV infected patients was a correlation between the concentration of ssDNA in liver tissue and sFas in serum (r=0.52; p<0.02) observed.

No correlation between CD4+, CD8+ counts, HCV, HIV viraemias, and ssDNA in liver tissue of HCV and HIV infected patients was observed – *Tab. 2*.

***Figure 4*. The correlation between ssDNA in liver tissue and the concentration of sFas among HCV and HIV infected patients**



Discussion

It is argued that HBV stimulates apoptosis in hepatocytes stronger than HCV [2]. Strong stimulation of such process by HBx antigene of HBV viruses can be one of the reasons. In liver tissue of HCV infected patients, Mutluat et al. [3] detected higher concentration of Bcl-2 in comparison to those infected with HBV. Bcl-2 inhibits apoptosis, which can be another reason for the stronger course of programmed cell death processes among patients infected with HBV. In own studies, the highest concentration of ssDNA in liver tissue was observed among HBV infected patients. However, no significant difference between HBV and HCV infected patients was observed. This result suggests that programmed cell death activations by HBV as well as HCV are comparable. In the studies by Bondini and Younossi [4], it was shown that HCV genotype 3 stimulates apoptosis stronger than genotype 1. Also in own studies, the concentration of ssDNA among patients infected with HCV genotype 3 was higher in comparison to those infected with genotype 1. These observations can explain biological differences as well as immunity to antivirus treatment due to HCV genotype 1 or 3 infection.

HIV infection activates apoptosis of CD4+ lymphocytes, but not of hepatocytes [5]. HCV and HIV co-infection, in comparison to HCV-infection alone, predisposes to a more frequent occurrence of primary liver neoplasms. Esposito et al. [6] argue that the reason for this can lie in reduced apoptosis of hepatocytes among HCV and HIV co-infected patients. In own studies, the concentration of ssDNA in liver tissue of HCV and HIV co-infected patients was lower in comparison to those infected with HCV or HBV. It seems that HIV inhibits the processes of apoptosis in liver tissue, either in a direct or indirect way. HCV and HIV co-infected patients with higher CD4+ count show a slightly stronger activity of programmed hepatocyte death processes (with no significant statistical difference). No HCV or HBV viraemia influence on programmed cell death processes was detected. In own studies it was shown that inflammatory activity and fibrosis intensification in liver tissue do not influence the concentration of ssDNA in HCV infected patients. In the group of HBV infected patients, lower inflammation and less intense fibrosis correlated with the concentration of ssDNA. These observations suggest that there is a relation between fibrosis in the liver and programmed cell death processes.

HBV and HCV viruses stimulate programmed cell death indicators such as TNF, Fas, TRAIL, IL-1 [7]. In own studies, the concentration of sFas among HBV infected patients was slightly higher in comparison to those infected with HCV or HCV and HIV. Macias et al. [8] showed that HCV and HIV infections cause a higher expression of Fas in hepatocytes. However, the correlation between the concentration of sFas in serum and the concentration of ssDNA in liver tissue was detected only among HCV and HIV infected patients. This undermines the importance of the assessment of sFas and sFasL concentrations as a programmed hepatocyte death indicatior among patients infected with HBV and HCV with no HIV co-infection.

Balasubramanian et al. [9] argue that the activity of apoptosis in hepatocytes among patients infected with HCV and HIV is regulated by the stimulation of Fas/FasL receptors; moreover, HCV E2 and HIV gp 120 stimulate the expression of caspase 3. In own studies, a higher concentration of ssDNA was present in patients with higher HCV and HIV viraemia. It seems that, in infections with these viruses, HIV viruses inhibit programmed cell death activity, as assessed by the concentration of ssDNA, either in a direct or indirect way.

Conclusions

HCV, HBV and HIV viraemias do not correlate with ssDNA concentration in liver tissue. In patients with HCV and HIV co-infection, CD4+ and CD3+ counts do not correlate with ssDNA concentration in liver tissue. HIV infection seems to inhibit apoptosis processes in liver tissue of HCV and HIV co-infected patients. In the case of HCV and HIV co-infection, the concentration of sFas in serum correlates with the concentration of ssDNA in liver tissue. This phenomenon is not observed among HBV or HCV infected patients.

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