Dysregulation of Apoptotic Death in the Pathogenesis of Virus-Induced Cytogenetic Instability of Blood Lymphocytes

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The cytogenetic status and activity of regulatory systems for stability of the cell genome were evaluated in patients with chronic viral persistence. Hepatitis B and C viruses damage the chromosome apparatus of peripheral blood lymphocytes. Cytogenetic instability of immunocompetent cells during chronic viral infection was associated with inhibition of DNA excision repair system and dysregulation of apoptosis in target cells.

Key Words: *chronic viral hepatitis; lymphocyte; apoptosis; chromosomal aberrations; DNA repair system*

One of the major recent achievements in medical and biological science is identification of the system, which provides elimination of abnormal and potentially dangerous cells from the organism. This system is responsible for monitoring of changes in key intracellular processes that cause or can cause dysfunction of the cytogenetic apparatus. Cell differentiation is temporally suppressed under these conditions. These changes result in repair of damages or apoptosis in cells.

The maintenance of genetic homeostasis in immunocompetent blood cells during infection is of particular interest. Most agents causing chronic viral infections produce damage to lymphocytes that are involved in antiviral protection [4,5]. Infection of blood lymphocytes determines long-term survival and dissemination of viruses in the macroorganism and causes various immunopathological reactions [5]. Virus-induced changes in nuclear structures contribute to an appropriate effector response of the cell.

Integration of the viral genome (*e.g.*, DNA molecule in hepatitis B virus) into the host cell genome can initiate virus-induced carcinogenesis. The virus inhibits poly-(ADP-ribosyl)-polymerase that serves as a key enzyme for regulation of gene interactions and repair of DNA and cell membrane. Moreover, the virus has a transactivating effect on the cyclin-dependent kinase inhibitor p21 [12]. Hepatitis C virus is a RNA-containing virus. Due to the presence of proteins, this virus inhibits kinase phosphorylation and impairs transcription and translation of key components in cyclin complexes E and D [15].

Genetic changes in immunocompetent cells play an important role in progression of chronic viral hepatitides and pathogenesis of cirrhosis and hepatocellular carcinoma. Much attention is paid to studying of the molecular mechanisms of correction of genomic changes in peripheral blood lymphocytes. Here we studied the role of lymphocyte apoptosis in the regulation of cytogenetic homeostasis in these cells during persistence of hepatitis B and C viruses.

MATERIALS AND METHODS

We examined 64 patients with moderate or mild chronic viral hepatitis (49 men and 15 women, 18-

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50 years). The diagnosis of viral hepatitis was made by the results of clinical examination (syndromes of cholestasis, cytolysis, and mesenchymal inflammation), liver ultrasonography, and scintigraphy. A morphological study of liver biopsy specimens revealed hepatitis with mild (3-8 points on the Knodell scale) or moderate inflammation (9-12 points on the Knodell scale). Etiological verification was made by blood tests for hepatitis B virus DNA (HBV), hepatitis C virus RNA (HCV, polymerase chain reaction), and serological markers of HBV (HBe antigen; HBs antigen; anti-HBcor IgM; and anti-HBcor total) and HCV (anti-HCV Ig for cor, C protein, and nonstructural proteins NS-3, NS-4, NS-5; and anti-HCV IgM). There were 22 patients with viral hepatitis B, 28 patients with hepatitis C, and 14 patients with mixed infection (B+C). The patients with alcohol dependence, drug abuse, and infectious or noninfectious inflammation were excluded from further observations. All patients were examined before the start of therapy. The control group included 17 sex- and age-matched healthy donors. The venous blood was taken from fasting patients in the morning. The blood was stabilized with 25 U/ml heparin.

The samples for lymphocyte chromosome analysis were prepared as described elsewhere [11]. The heparinized blood (1 ml blood, 25 U/ml heparin) was mixed with 3 ml complete culture medium containing 0.01 mg/ml phytohemagglutinin (Difco). Lymphocytes were cultured at 37°C for 52 h. Colchicine (0.25 ml, 0.01% solution, Fluka) was added to flasks with cultured lymphocytes 2 h before the end of culturing to obtain metaphase cells. The samples were stained with azure II and eosin for 10 min. One hundred metaphase plates were examined in each patient. We took into account the count of cells with chromosomal aberrations, as well as the number and type of chromosomal aberrations (according to classification [2]).

Activity of UV-induced DNA excision repair system in lymphocytes was estimated by means of scintillation radiometry [3]. The whole heparinized blood (50 μl) was irradiated with UV light for 15 sec. Unirradiated blood samples served as the control. The samples were incubated in a nutrient culture medium containing 10 μCi/ml [^{3}H]-thymidine at 37°C for 2 h. Radioactivity was measured using a Mark-3 scintillation counter. The intensity of DNA repair synthesis was determined by the index of stimulation. This index was calculated as the ratio between radioactivity of UV-irradiated and not irradiated samples (cpm).

The number of lymphocytes carrying apoptotic marker CD95 receptor was estimated immunocytochemically using Dako kits [6]. The suspension of isolated lymphocytes was incubated with mouse monoclonal antibodies to CD95 receptors for 60 min to identify specific antigens on the cell membrane. The samples were washed 3 times with phosphate buffered saline (pH 7.2-7.4) and incubated with binding biotinylated antibodies for 30 min. The sample was washed 3 times with phosphate buffered saline, coated with alkaline phosphatase-conjugated streptavidin, and repeatedly washed. The samples were incubated with cold chromogen substrate Fast Red for 10 min. The chromogen was washed out with distilled water. The samples were stained with hematoxylin (Sigma). Light microscopy under oil immersion included counting of positively stained cells (per 200 lymphocytes).

Apoptosis in lymphocytes was identified by phosphatidylserine expression on the outer cell membrane. This study involved FITC-labeled annexin V (Caltag) [14]. Mononuclear leukocytes (2×10^6 cells/ml) were isolated on a Ficoll-Paque density gradient (Pharmacia) and cultured in a complete nutrient medium containing 90% RPMI 1640, 10% inactivated fetal bovine serum, 0.3 mg/ml L-glutamine, 10 mM HEPES (Flow), and 100 μg/ml L-glutamine at 37°C and 5% CO_2 for 24 h. After incubation the cells (10^6 cells/ml) were suspended in annexin V-FITC-containing buffer (Caltag) for 10 min and analyzed by flow cytofluorometry on an Epics XL cytometer. Green fluorescence (FITC, 530 nm) was recorded in the lymphocyte gate.

The data distribution was estimated by Kolmogorov—Smirnov test. The results were analyzed by Student's t test (Gaussian distribution data) and Mann—Whitney U test (non-Gaussian distribution data). The differences were significant at $p<0.05$.

RESULTS

Analysis of metaphase plates showed that the number of aberrant lymphocytes increases in the peripheral blood from patients with chronic hepatitides B, C, and B+C. Structural changes in chromosomes were mainly presented by chromatid aberrations (single fragments, Table 1). The number of hyperploid cells increased only in patients with chronic hepatitis C (Table 1). Previous studied showed that chronic hepatitides B and C are accompanied by variations in cytogenetic indexes (*e.g.*, chromatid and chromosomal breaks) [1]. The proposed mechanisms for cytogenetic changes include integration of the viral genome into immunocompetent cell DNA, release of lysosomal enzymes, and activation of free radical oxidation of proteins, lipids, and nucleic acids.

TABLE 1. Chromosome Aberrations in Peripheral Blood Lymphocytes from Patients with Chronic Viral Hepatitides (%, $X \pm m$)

Parameter	Healthy donors (n=17)	Hepatitis B (n=22)	Hepatitis C (n=28)	Hepatitis B+C (n=14)
Aberrant cells	1.28±0.35	2.70±0.33*	2.50±0.46*	2.90±0.43*
Chromatid aberrations	0.66±0.19	2.20±0.25*	1.80±0.44*	2.10±0.41*
single fragments	0.66±0.19	2.00±0.26*	1.62±0.32*	2.10±0.41*
isochromatid exchanges	N. d.	Singl	Singl	N. d.
Chromosomal aberrations	0.65±0.20	0.50±0.16	0.25±0.16	0.70±0.15
paired fragments	0.65±0.20	0.50±0.16	0.25±0.16	0.70±0.15
ring chromosomes	N. d.	N. d.	N. d.	N. d.
dicentric chromosomes	N. d.	N. d.	N. d.	N. d.
Hyperploid cells	N. d.	N. d.	0.63±0.26*	Singl

Note. Here and in Table 2: *p<0.05 compared to healthy donors. N. d.: not detected.

The mutagenic effect of viral factors on host cells is determined not only by the type and degree of chromosome aberrations, but also by activity of repair systems maintaining cytogenetic stability [3]. Radiometry showed that the efficiency of excision DNA repair was reduced in all patients (Table 2). Published data suggest that deceleration of the repair process results from adverse effects of viral proteins on the interaction of p53 with the complex of TFIIH nucleotide excision repair [12]. Moreover, HBx can directly interact with damaged sites in cell DNA, which prevents nucleotide substitution and recovery of breaks by the repair system [13].

Programmed cells death is initiated upon hypoactivity of the repair system and presence of genomic damage [7]. Many viruses modulate the regulation of intracellular homeostasis and affect the balance between growth and apoptotic capacities of infected cells. The number of lymphocytes carrying the CD95 receptor increased in chronic hepatitis C patients (Table 2). Our results are consistent with the general concept that the FasL/Fas system plays a role in cytotoxic elimination of virus-infected cells. The process can be accompanied by failure of compensatory mechanisms of immune regulation and prevalence of antiapoptotic viral activity over the protective cellular response. These changes were probably typical of hepatitis B and B+C patients with an intermediate number of Fas^+ lymphocytes (near-normal level, Table 2). Activation of programmed cell death requires binding of the Fas receptor to various specific ligands, including Fas-L in cytotoxic T lymphocytes and natural killer cells, several cytokines, and antigens [7].

Cell death is realized via receptors or occurs under the influence of viral factors on key regulatory molecules of the cell cycle [9]. The annexin test for apoptosis in blood lymphocytes showed that the number of apoptotic cells decreases in hepatitis C patients (Table 2). However, apoptosis remained practically unchanged in hepatitis B patients (Table 2). Similar apoptotic response of lymphocytes from patients with hepatitides C and B+C suggests that hepatitis virus C is more potent than hepatitis B virus in preventing cell death.

Strong experimental evidence exists that hepatitis C virus produces different antiapoptotic effects. The nonstructural NS5A protein of hepatitis C virus can inhibit apoptosis in infected cells, which is associated with its binding to phosphoinositol-3-

TABLE 2. Index for Stimulation of the Excision DNA Repair System and Number of Fas^+ and Apoptotic Lymphocytes in Patients with Chronic Viral Hepatitides ($X \pm m$)

Group	Index for stimulation of the excision DNA repair system, arb. units	Number of $CD95^+$ lymphocytes, %	Number of apoptotic lymphocytes, %
Healthy donors (n=17)	1.91±0.15	10.83±1.13	11.39±1.27
Hepatitis B patients (n=22)	1.12±0.12*	10.84±1.14	8.89±1.65
Hepatitis C patients (n=28)	1.16±0.10*	16.40±1.72*	6.32±1.43*
Hepatitis B+C patients (n=14)	1.10±0.11*	1.67±2.79	6.01±0.85*

kinase p85 regulatory subunit. Activation of this enzyme results in phosphorylation of the proapoptotic cell protein Bad. NS5A prevents activation of caspase-3 and release of cytochrome C from mitochondria of cultured HepG2 cells [8]. The antiapoptotic effect of HCV-core protein is manifested in activation of ICAD protein (caspase-activated DNase inhibitor), which prevents DNA fragmentation [15].

Modulation of cells death by hepatitis B virus is probably mediated by other mechanisms. Previous studies showed that viral HBx protein protects infected cells from Fas-mediated apoptosis by inducing NF-kB [13]. Under certain conditions this protein sensitizes the cell to apoptosis. This effect is related to the induction of Myc protein expression. HBx causes aggregation of mitochondria at the peripheral region of cells, decrease in the mitochondrial potential, and release of cytochrome C from the cytosol, which results in cell death. These changes are associated with the ability of amphipathic α-helixes to incorporate into the mitochondrial membrane with the formation of transmembrane pores [10].

Our results suggest that viral infections are accompanied by insufficiency of the systems for monitoring and maintenance of genetic stability at the molecular (DNA repair assay) and cellular levels (apoptosis assay). These changes result in the phenomenon of genomic instability. The virus (genotoxic factor) determines a significant increase in the number of genetically defective immunocompetent cells. Functional deficiency of these cells contributes to several immunopathological reactions. It should be emphasized that chronic infection can result in exhaustion of the cellular system and, therefore, shortening of the mitotic cycle. Therefore, the number of unrepaired defects with high carcinogenic activity increases in active chromatin.

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