Molecular Mechanisms of Changes in TNF-α Production by Blood Mononuclears in Chronic Viral Hepatitis C

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Chronic viral hepatitis C is associated with decreased production of TNF-α by the peripheral blood mononuclear leukocytes irrespective of virus genotype and degree of the morphological activity of the process in the liver. This process positively correlates with the increase in the content of TNF-α soluble receptor (molecular weight 55 kDa), which can play a role in the mechanisms of immunopathogenesis of long persistence of hepatitis C virus in the body.

Key Words: *chronic viral hepatitis; hepatitis C virus; TNF-α; TNF-α soluble receptor*

Chronic viral hepatitis C (CVHC) is a pressing problem of modern medicine, because of its high prevalence in the working population (according to WHO data, about 3% of the population are infected with hepatitis C virus — HCV), progredient course, and inefficiency of modern methods of antiviral therapy. Virus persistence and chronic course of HCV are fraught with the development of severe clinical complications: decompensated cirrhosis of the liver and hepatocellular carcinoma [5,8-10,14].

Hepatitis C virus is characterized by high variability of antigens, molecular and genetic variability of isolates, and often induces the development of latent infection. Due to modulation of the genetic structure the virus protects itself from the host immune system and adapts to exogenous inhibitors of different stages of its vital cycle. Some reports persuasively demonstrated that mutant strains play an important role in HCV persistence and progress of acute and chronic hepatitis [8,15].

The direct cytopathogenic effect of HCV causes cytolysis and clearance of infected cells. This leads to activation of T-effector and phagocytic immune systems, the main producers of cytokines largely regulating the triggering, direction, and realization of the antiviral immune response [14].

The TNF family (ligands and receptors) plays a key role in the regulation of the immune response. TNF-α is a multifunctional cytokine characterized by pronounces pleiotropism. It is produced in low amount by resting cells, but becomes one of the main factors released by activated macrophages and lymphocytes. This cytokine plays the leading role in the development of local and general systemic inflammatory processes and infectious diseases, in particular in viral infections [2-4,7]. TNF-α regulates the intensity of inflammation, immune response, activates T- and B-lymphocytes, natural killers, exerts hepatotoxic and pyrogenic effects, is involved in apoptosis, cell proliferation, improves resistance to infectious factors. The TNF-receptor system is involved in the network of chemokines stimulating attraction of immune cells in infectious foci [1,11]. The 26-kDa transmembrane form of pro-TNF-α, precursor of mature cytokine, is located on the plasma membrane and then is proteolytically cleaved by metalloproteinases with the release of mature 17-kDa TNF-α [4].

Realization of the biological effects of TNF-α is possible after its binding to specific high affinity re-

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ceptors TNF-R1 and TNF-R2 (molecular weights 55 and 75 kDa, respectively). The role of membrane-associated receptors in the realization of activation signals received from the ligand is discussed on a wide scale [1,6,12], but the mechanism of cell-cell cooperation of blood immunocompetent cells in the development of chronic infectious process cannot be completely understood without investigating the role of soluble receptors. The production and content of soluble receptors in the serum and biological fluids reflect the functional status of the organism. These parameters are changed in cancer, autoimmune diseases, and infections of different etiology.

Despite the obvious role of TNF system in the mechanisms of chronic transformation of viral infections, the significance of TNF-α and its receptor system in the pathogenesis of chronic viral hepatitis remains not quite clear. We studied the production of TNF-α and its soluble receptor by the peripheral blood mononuclear cells (MNC) in patients with CVHC depending on the virus genotype and degree of inflammatory process activity in the liver.

MATERIALS AND METHODS

The study was carried out in 45 patients (31 men and 14 women aged 18-45 years) with CVHC of mild and moderate activity of the process in the liver. HCV genotype 1b was detected in 28 patients, 2a and 3a genotypes in other patients. The mean duration of viral infection was 6±2 years (1.5-12 years). The disease was diagnosed on the basis of clinical, epidemiological, instrumental (scintigraphic, ultrasonic, and morphological analysis of the liver), serological (enzyme immunoassay of anti-HCVcor IgG and anti-HCVns3.5), and PCR findings. All patients were examined during virus replication. Exclusion criteria were ethanol and narcotic dependence and infectious and inflammatory processes of etiology other than HCV. Control group consisted of 20 age- and sex-matched donors. Venous blood was collected after overnight fast and stabilized with heparin (25 U/ml).

The concentrations of TNF-α and its soluble receptor (rTNF-55P) were measured in MNC culture supernatants. Mononuclear cells (2×10^6 cell/ml) isolated on Ficoll-Paque density gradient (Pharmacia) were cultured in RPMI-1640 with 10% inactivated FCS, 0.3 mg/ml L-glutamine, 10 mM HEPES (Flow), 100 μg/ml gentamicin at 5% CO_2 for 24 h. The production of TNF-α and rTNF-55P was stimulated by adding phytohemagglutinin (PHA) (Difco). The content of TNF-α and rTNF-55R in the supernatants was measured by enzyme-linked immunosorbent assay (Cytimmune, Procon). Optical density was evaluated on a microplate photometer Multiscan EX (Thermo-LabSystems). The concentrations of TNF-α and rTNF-55R were estimated by calibration curves.

The structure of lymphocyte plasma membrane was investigated using pyrene probe. Lymphocytes were isolated on Ficoll-Verografin density gradient (1.077 g/cm^3). The resultant lymphocyte suspension was diluted with Hanks' solution to a concentration of 2×10^5 cell/ml. Lipotropic probe (pyrene) in a concentration of 10 mM was added to the lymphocyte suspension and the mixture was incubated for 20 min. The eximer/monomer fluorescence ratio (470/390 nm) was evaluated at excitation wavelength of 285 nm.

The normal character of distribution was evaluated using the Kolmogorov—Smirnov test, the equality of selected means was tested using Student's *t* test (for normal distribution) and Mann—Whitney's *U* test (if the distribution deviated from the normal). Analysis of correlations was carried out by calculating Spearman's *r* coefficient.

RESULTS

Special attention was paid to HCV genotyping: 7 genotypes and more than 160 HCV subtypes were identified; their incidence varies in different geographic regions. A relationship exists between the route of infection and HCV genotype: 1b genotype is more often associated with hemotransfusions, medical manipulations, and occupational infection; 3a genotype is associated with intravenous drug use; and 2a genotype is more often diagnosed in co-infection with HBV [13]. Attempts at detecting the relationship between HCV genotype and morphological activity or severity of fibrosis in CVHC patients failed, though retrospective studies aimed at detection of HCV genotypes in patients with liver cirrhosis and hepatocellular carcinoma clearly demonstrated the highest incidence of 1b genotype [14]. We also failed to detect a clear-cut relationship between the capacity of peripheral blood MNC to produce TNF-α and HCV genotype. Spontaneous and PHA-stimulated *in vitro* production of TNF-α by MNC did not differ statistically in patients with different virus genotypes (Table 1).

The status of immune response mediators in patients with chronic viral hepatitis, specifically, produc-

TABLE 1. Spontaneous and PHA-Stimulated *In Vitro* Production of TNF-α by MNC from Patients with CVHC Depending on Virus Genotype ($\bar{X}\pm m$)

TNF-α concentration, pg/ml	Genotype	
	1b	2a and 3a
Spontaneous	40.00±5.97	37.50±6.33
Stimulated	107.75±21.49	89.51±9.41

tion of TNF-α, characterizes the inflammatory process in the liver and eventually determines the rate of its development [7,9,11]. With this fact in mind, we divided patients with CVHC into 2 groups with different activity of the pathological process in the liver. Constitutional and PHA-induced *in vitro* production of TNF-α by MNC was decreased significantly in patients with CVHC of low and moderate activity. The mean level of PHA-stimulated *in vitro* production of TNF-α, reflecting the reserve potentials to secrete this cytokine, was 45% higher in CVHC patients with moderate activity of the process in comparison with patients with low activity (Table 2). TNF-α plays an important role in the formation of specific immune response developing during long-term persistence of pathogens (carriers of foreign genetic information), therefore the deficiency of this cytokine can promote the development of imbalance in the production of immunoregulatory Th-1/Th-2 cytokines. This can result in polarization of the immune response (impairment of phagocytic, cytotoxic activities of the cells and apoptosis induction), ineffective elimination of the virus from the body, and development of chronic form of the disease. Published reports about the production of TNF-α in viral hepatitis are rather contradictory. Elevated serum levels of this immunoregulatory cytokine were detected in CVHC patients [2,10, 11,14]. The level of TNF-α changed little in comparison with the control group [3]. On the other hand, other authors reported that MNC of CVHC patients released less TNF-α *in vitro* in comparison with normal human MNC [12].

One more possible mechanism of the decrease of TNF-α level in culture medium in patients with CVHC is increased number of cytokine "traps" (soluble forms of their receptors) [1]. We measured soluble TNF-α receptor with molecular weight of 55 kDa in supernatants of mononuclear leukocyte cultures. Analysis of the results showed that CVHC was associated with an increase in the constitutional and PHA-stimulated *in vitro* production of rTNF-55R (Table 2). Basal production of rTNF-55R was significantly higher in CVHC patients with low activity in comparison with patients with moderate pathological activity of the hepatic process (Table 2). Decreased capacity of immunocompetent cells to produce TNF-α positively correlated with increased content of rTNF-55R (r=0.618, p<0.05 and r=0.702, p<0.05, respectively).

Lymphocyte activation, along with expression of membrane receptors, was also associated with production of soluble receptor molecules — potent regulators of cytokine activity. Extracellular receptor domains

TABLE 2. Spontaneous and PHA-Stimulated *In Vitro* Production of TNF-α and rTNF-55P by MNC from Patients with CVHC Depending on Activity of the Inflammatory Process in the Liver ($\bar{X}$±m)

Parameter	Donors	Patients with CVHC, activity	
		mild	moderate
TNF-α concentration, pg/ml			
spontaneous	103.65±16.22	36.70±3.37*	39.48±2.92*
stimulated	342.85±60.86	66.55±5.30*	96.70±11.49*$^{+}$
rTNF-55P concentration, pg/ml			
spontaneous	5.56±1.38	70.01±20.03*	34.02±8.24***$^{++}$
stimulated	8.89±2.58	22.50±5.61***	26.07±7.08***

Note. Here and in Table 3: *p<0.001, **p<0.01, ***p<0.05 compared to donors; ^{+}p<0.05, ^{++}p<0.01 compared to patients with mild CVHC activity.

TABLE 3. Results of Fluorescent Probing (l=285 nm) of Lymphocyte Plasma Membrane in CVHC Patients with Different Activity of Pathological Process in the Liver (X±m)

Parameter	Donors	Patients with CVHC, activity	
		mild	moderate
Fluorescence intensity, arb. units			
I_{470}/I_{370}	1.373±0.019	1.099±0.024*	1.195±0.013**
I_{470}/I_{390}	1.400±0.012	49.23±2.17***	47.30±2.58**
Extent of energy migration from tryptophan to pyrene, R%	59.48±3.05	49.23±2.17***	47.30±2.58**

proteolytically cleaved from the surface of cell membranes can bind to free cytokine molecules outside the cell, preventing cytokine association with membrane receptors directly on the target cell. The signaling is thus impaired and the realization of the effect prevented. Soluble receptors can also function as competitive antagonists. They act as cytokine transmitters protecting these agents from protease destruction and prolonging their life span in systemic circulation, and participate in cytokine transport to the focus of injury and their elimination from the body [1,10,13].

The causes of increased concentrations of soluble cytokine receptors in biological fluids are now discussed. According to many studies, shedding of proteins from cell surface as a result of destructive processes in the plasma membrane (LPO intensification, resulting in modified orderliness and microviscosity of the membrane lipid bilayer, leading to modification of their structural characteristics) can play the key role in increase in the content of soluble TNF-α receptor. Increased microviscosity of the lymphocyte plasma membrane, detected by means of fluorescent lipotropic probe (pyrene), confirms this mechanism (Table 3).

Increased shedding of the receptor from cell membrane surface can be induced by the virus [1,12]. Among the possible mechanisms of increased concentration of soluble receptors of cytokines are overexpression of alternatively spliced mRNA of mononuclear leukocytes, dissociation of cytokine-receptor complexes [6].

Hence, CVHC is associated with decreased production of TNF-α by the peripheral blood mononuclear leukocytes irrespective of the virus genotype and activity of the pathological process in the liver. Decreased production of TNF-α by peripheral blood mononuclear leukocytes positively correlates with increased content of soluble rTNF-55R receptor, which can contribute to the mechanisms of immunopathogenesis of long persistence of HCV.

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