

The role of NK cells in pathogenesis of thrombocytopenia in hepatitis C infection

Rola komórek natural killer (NK) w patogenezie małopłytkowości w przebiegu zakażenia wirusem C (HCV)

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Abstract

Aim: The aim of this study was to discuss the pathomechanism of thrombocytopenia in HCV infection.

Material and methods: Twenty-four HCV positive patients with thrombocytopenia (HCV-TP) and twelve HCV positive patients with normal count of platelets (HCV-NP) as a control group were enrolled in this study. Subpopulations of lymphocytes and serum platelet-associated immunoglobulin (PAIg) levels were determined and correlation analyses were performed.

Results: The percentages of T lymphocytes (mainly CD8+) were higher in the HCV-NP group than in the HCV-TP group ($p < 0.04$). There were no differences in lymphocyte B (CD19+) levels between patients with HCV-TP and HCV-NP. Elevated titres of PAIg were detected in 14/24 (58.3%) of HCV-TP cases, whereas PAIg were negative in the HCV-NP patients. There was not a relationship between severity of thrombocytopenia and serum PAIg. A significant decrease in NK cells (79.1%) was observed in the HCV-TP group compared with the HCV-NP group (58.3%). Moreover, NK cells were not detected in 11 patients from the HCV-MP group (45.8%) and in 1 patient from the HCV-NP group (8.3%) ($p < 0.004$, $p < 0.04$). Correlation analyses demonstrated a significant negative correlation between NK cells count and PAIgG levels ($p < 0.005$). We conclude that thrombocytopenia in our HCV-positive patients appears to be autoimmune mediated.

Conclusions: The above findings show a direct correlation between deficient NK cells and PAIg level and suggest an important role of NK population as a mechanism contributing to thrombocytopenia in HCV-infected patients.

Streszczenie

Cel pracy: Celem pracy była próba wyjaśnienia patogenezy małopłytkowości u chorych z przewlekłym zapaleniem wątroby typu C (HCV).

Materiał i metody: Badaniem objęto grupę 24 chorych HCV-dodatnich ze współistniejącą małopłytkowością (HCV-TP). Grupę kontrolną stanowiło 12 chorych HCV-dodatnich z prawidłową liczbą płytek krwi.

Wyniki: W obu badanych grupach średni odsetek limfocytów T i B pozostawał w granicach normy, chociaż u chorych HCV-TP wykazano obniżony odsetek limfocytów T (CD8) w porównaniu z grupą kontrolną ($p < 0,04$). Nie stwierdzono istotnych statystycznie różnic pomiędzy grupami w zakresie liczebności pozostałych subpopulacji limfocytów T i B. W grupie HCV-TP przeciwciała przeciw płytkowe wykazano u 14 chorych (58,3%), nie wykazano ich obecności w grupie kontrolnej. Nie stwierdzono zależności pomiędzy stężeniem przeciwciał a liczbą płytek krwi w grupie chorych z HCV-TP. W grupie HCV-TP u 19 chorych (79,1%) wykazano obniżony odsetek komórek NK, a u 11 chorych (45,8%) komórek tych nie stwierdzono. W grupie kontrolnej obniżony odsetek komórek NK dotyczył 7 chorych (58,3%), w jednym przypadku komórek NK nie wykryto (8,3%). Różnice te były istotne statystycznie ($p = 0,004$, $p = 0,04$). Wykazano istotną ujemną korelację między liczbą komórek NK a mianem PAIgG ($p < 0,005$). Wyniki powyższych badań dowodzą, że małopłytkowość w przebiegu zakażenia HCV może mieć charakter autoimmunologiczny.

Wnioski: Wykazana zależność między deficytem komórek NK a obecnością przeciwciał przeciw płytkowych sugeruje istotną rolę tej populacji komórek w patogenezie małopłytkowości w przebiegu przewlekłego zakażenia HCV.

Introduction

The long-term observations of non-A non-B hepatitis cases were crowned in 1989 by discovering type C virus (HCV). Thanks to progress in molecular biology, a team of scientists from Chiron Corporation in California achieved this success. It was the beginning of studies on the aetiology and pathogenesis of HCV infections, whose immunological implications are an integral part of the clinical picture of the illness. There are an estimated 170 million infected in the world, while in the USA alone 38 000 get infected every year [1].

HCV infection is mostly gentle, sometimes even without visible lab symptoms. According to various authors 50-70% of cases go on to a lingering infection. The hepatocyte is the main, but not the only, target cell for HCV. Many well-documented papers have indicated the presence of the virus in mononuclear cells of blood, T and B lymphocytes, bone marrow cells, including megakaryocytes and platelets [2-4]. The ability to replicate in many organs and lingering infection cause HCV to indicate a particular susceptibility to induction of extrahepatic syndromes, which coexisting with hepatitis can become a major clinical complication and often change the prognosis.

A particularly frequent complication of HCV infection is thrombocytopenia (HCV-TP). Its frequency varies from 10 to 71% according to miscellaneous reports [3, 5, 6]. Studies on patients diagnosed as immune thrombocytopenic purpura (ITP) have proven a significantly more frequent presence in HCV-positive patients (22-30%) than in the healthy population (0.4%) [7, 8]. The phenomenon's mechanism has not yet been revealed. The mechanisms through which HCV may induce thrombocytopenia remain unclear. The aim of the study was an attempt to explain the pathogenesis of thrombocytopenia in patients with a lingering HCV infection.

Material and methods

Twenty four patients with chronic HCV infection and coexisting thrombocytopenia (PLT <100 G) – (HCV-TP) were enrolled in this study. There were 8 women and 16 men with a median age of 51.7 years (range 18-76 years). The control group consisted of 12 patients infected with HCV, with a normal platelet count (HCV-NP): 4 women and 8 men with a median age 46 years (range 27-70 years). Patients without splenomegaly and with a normal count of reticulocytes were qualified for the research. Patients with coexisting autoimmune disorders and/or cancers, or earlier treated with interferon, were excluded.

Presence of the virus (HCV-RNA) was confirmed by polymerase chain reaction (PCR). Quality and quantity cytological estimation of megakaryopoiesis was carried out on the basis of bone marrow biopsy. Smears were routinely stained by the Pappenheim (May-Grunwald-Giemsa) method and analyzed under a light microscope. Quantity estimation of the B, T lymphocyte population and the NK cells in the blood was analyzed by flow cytometric techniques, using specific monoclonal antibodies. Quantity and quality estimation of the antiplatelet antibodies (PAIg) was executed in direct and indirect immunofluorescence tests, using technology that integrates flow cytometry (CD-41 IPE) with immunofluorescence microscopy. To achieve this a number of resources were used: a fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson) Cellquest 3.1 Software computer program, the length of a light wave for the green fluorescence ranging from 515-548 nm, a 105 fluorescence detector and an analysis of 10 000 platelets. A bright cell's percentage was registered on histograms (canals ranging from 0-255) for a gate set always by side scatter (SSC) in a fluorescence microscope in 400 × magnification. The visible fluorescence was considered a positive reaction and the light intensity was determined on a 1+ to 3+ scale. It indicated level of PAIg in the patient's serum according to the scale below: (-) below 30% activated cells, (+) 30-50% activated cells, (++) 50-80% activated cells, (+++) 80-100% activated cells.

Statistical analysis

The statistical analysis was carried out with the computer program Statistica (StatSoft Inc., USA, v. 6.0). The accordance of sequences' variables in every group with a normal disposition was checked with a Shapiro-Wilk normality test. Arithmetical averages and standard deviation were estimated. Due to the fact that most analyzed data did not have a normal distribution, the comparison in the lymphocyte subpopulation (absolute values) between the studied group and the control group was carried with a nonparametric Mann-Whitney test. The quantity comparison in every subgroup was estimated using a two-way chi square test with Fisher's correction. For the dependency between PAIg level and the platelet count, p value and Spearman rank correlation factors were determined. A p value less than 0.05 was considered a significant difference between subgroups.

Results

A percentage income above the megakaryocyte norm was observed in 10 patients (41.6%) in the 24 HCV-TP patients; in 1 case there were no megakaryocytes found in the bone marrow sample (4.2%); and in the remaining 13 patients (54.2%) the megakaryocyte level was normal.

In the control group (HCV-NP) there were no deviations concerning megakaryopoiesis.

The average count of B lymphocytes (CD19+) remained normal in both observed groups. There was a lymphocyte B percentage income observed in 1 case in the HCV-TP group (4.1%) and in 1 case in the control group (8.2%). A lymphocyte B percentage drop under the norm was observed in 3 patients (12.5%) in the HCV-TP group and in 1 patient from the control group (8.2%). Differences between observed groups were statistically insignificant (Table I).

The average number of T lymphocyte (CD4 and CD8) was normal in both observed groups. In the HCV-TP group a significantly lower percentage of T lymphocyte was observed, mainly CD8 lymphocytes, compared to the HCV-NP group ($p < 0.04$). In this group in 3 cases (12.5%) a CD4/CD8 value decrease was observed, caused mostly by an increase of CD8+ lymphocytes in 2 patients (8.3%) and in 2 cases (8.3%) an additional CD4 lymphocyte subpopulation percentage decrease.

In the HCV-NP group there was a decrease of CD4/CD8 value in 3 patients (25%) and it was a result of a CD8 lymphocyte percentage increase in 3 cases (25%) and a CD4 lymphocyte subpopulation value decrease (16.6%). These differences were statistically insignificant between the studied patient groups (Table II).

An NK cell (CD56+/CD3-) percentage decrease was observed in both studied groups. The average NK cell percentage count for the HCV-TP group was 3.9% and for the HCV-NP group 7.9%. In 19 patients in the HCV-TP group (79.1%) a decreased percentage of NK cells was observed; none were spotted in 11 cases (45.8%).

A decreased percentage of NK cells was observed in 7 patients in the HCV-NP group (58.3%); none were spotted in 1 case (8.3%). These differences were statistically significant (respectively $p < 0.03$, $p < 0.04$) (Table III).

Antiplatelet antibodies (PAIg) were observed in a direct test in 12 patients in the HCV-TP group (58.3%), including IgG class antibodies (58.3%) and IgM (37.5%). IgG and IgM antibodies were present with equal frequency (4 cases, 16.6%) in an indirect test. There were no cases with PAlg spotted in the HCV-NP group. No significant correlation between PAlg levels and the platelet count in patients with HCV-TP was revealed.

Among 11 out of 24 patients from the HCV-TP group (45.8%), in which no NK cells (CD56+/CD3-) were

Table I. Lymphocyte subpopulations

Tabela I. Subpopulacje limfocytów we krwi obwodowej

	N	Lymphocytes		CD3		CD4		CD8		CD19	
		K/nl	%	K/nl	%	K/nl	%	K/nl	%	K/nl	%
HCV-TP	24	1990	66	1394	44	900.8	27.3	546.8	10.9	206.5	
	SD	(916)	(12.2)	(817.4)	(9.9)	(535.9)	(7.8)	(310.8)	(5.0)	(103.3)	
HCV-NP	12	2571	53.4	1824	29	993	23.0	785.8	11.3	388.0	
	SD	(585)	(15.4)	(528)	(10.7)	(368.2)	(9.6)	(329)	(8.2)	(280.6)	
p		<0.04	ns	<0.04	ns	ns	ns	<0.04	ns	ns	

Table II. Alterations of lymphocyte subpopulations

Tabela II. Odchylenia od normy w subpopulacjach limfocytów krwi obwodowej

	n	CD4		CD8		CD19		CD4/CD8	
		increase	decrease	increase	decrease	increase	decrease	increase	decrease
HCV-TP	24	1 (4.1%)	2 (8.3%)	2 (8.3%)	1 (4.1%)	1 (4.1%)	3 (12.5%)	1 (4.1%)	3 (12.5%)
HCV-NP	12	3 (25%)	3 (25%)	3 (25%)	3 (25%)	3 (25%)	3 (25%)	3 (25%)	3 (25%)
p		ns	ns	ns	ns	ns	ns	ns	ns

Table III. NK cell count (CD56+/CD3-) in study and control groups*Tabela III.* Liczba komórek NK (CD56+/CD3-) w grupie badanej i kontrolnej

	n	Average percentage CD56+/CD3-	Absolute values A CD56+/CD3-	Decreased values CD56+/CD3-	CD56+/CD3-==0
HCV-TP	24	3.9%	80.69	19 (79.1%)	11 (45.8%)
HCV-NP	12	7.9%	200.23	7 (58.3%)	1 (8.3%)
p		p=0.0236	p=0.0038	p=0.247	p=0.030

revealed, 10 cases carried PAIgG, shown in a direct test (90.9%), 5 cases had PAIgM class (45.4%) and 2 cases carried both, shown in an indirect test (18.2%).

Among 13 out of 24 patients from the HCV-TP group (54.2%), in which no NK cells were revealed, 4 cases carried PAIgG class, shown in a direct test (30.7%), while 3 carried PAIgM (23%). In this subgroup, PAIg were spotted in an indirect test both in IgM and IgG classes in 2 cases (15.3%).

In the matter of PAIg, shown in a direct test in the IgG class, these differences were statistically significant ($p < 0.005$) (Table IV).

Discussion

HCV possesses the ability to induce autoimmune reactions. In HCV infection benignity many well-documented auto-antibodies are observed, including cryoglobulins, anti-smooth muscle antibodies (SMA), antinuclear antibody (ANA), anti-mitochondrial antibodies (AMA), liver-kidney microsome antibody (LKM), anticardiolipin antibodies (ACA) and antiplatelet antibodies (PAIg) [9–13].

In most cases antibody level is low, with no significant clinical consequence. However, sometimes they are the main causative factor for autoimmune diseases, induced by HCV infection presence [11]. Elevated titres of PAIg were observed in 64–88% of patients with chronic hepatitis C [3, 12].

In the studied population PAIg were spotted in 58.3% of patients infected with HCV-TP, while none were found in any chronic HCV infection, which had the normal platelet count. However, there was no significant

correlation observed between PAIg levels in the serum and the platelet count. Other authors had similar observations [12, 14]. There are however reports which indicate a relationship between PAIg concentration and severity of thrombocytopenia in patients infected with HCV.

Nagamine, while estimating a 368 HCV infected population, observed thrombocytopenia in 41% cases and an 88.1% rate of elevated titres of PAIg presence. A significant negative correlation between PAIg and platelet count was described in this thesis. There was a significant negative correlation between PAIg titres and platelet counts [3]. Similar results were published in other reports [15] and they are the basis of the hypothesis that thrombocytopenia in an HCV infection is mostly autoimmunological in nature.

Some interesting observations were made by Rajan and coworkers, while conducting a comparison between groups of HCV-TP patients with adult chronic immune thrombocytopenic purpura (CITP). The authors observed no specific PAIg in the HCV-TP group, despite the fact that often there were other antibodies observed in those patients: ANA (42%), ACA (62%) and cryoglobulins (90%). It was found that thrombocytopenia in those cases can be a result of bonding nonspecific antibodies with platelets, which are then destroyed in the phagocytosis mechanism [8]. Thrombocytopenia could be a result of an "innocent bystander" effect. No relation between PAIg concentration and platelet count could explain the functional Fc macrophage receptor defect, observed in some lingering HCV infection patients. These observations suggest that annihilating platelets, covered by antibodies, depends not only on class and level of

Table IV. Antiplatelet antibodies (PAIg) and the CD56+/CD3- count in the HCV-TP group*Tabela IV.* Przeciwciała przeciw płytkowe a liczba CD56+/CD3- w grupie badanej

N	Direct Abs		Indirect Abs		
	IgG	IgM	IgG	IgM	
HCV-TP CD56+/CD3-==0	11	10 (90.0%)	5 (45.4%)	2 (18.2%)	2 (18.2%)
HCV-TP CD56+/CD3->0	13	4 (30.7%)	3 (23%)	2 (15.3%)	2 (15.3%)
p		p=0.004	ns	ns	ns

antibodies, but also on reticulo-endothelial system efficiency [15].

Quantity estimation of the T and B lymphocyte subpopulations did not reveal any differences between the HCV group and the control group. However, the results of the NK cell quantity study are interesting. 79.1% of cases in the HCV-TP group had decreased percentage of NK cells, and 45.8% had none. We found a significant correlation between low NK cell count and high PAIgG level ($p=0.004$). In over 90% of HCV-TP patients who had no NK cells, there were PAIg found in a direct test. Such a high PAIg titre percentage in patients with decreased NK cell count suggests a close pathogenetic relation between HCV infection, NK cell count and PAIg production.

The NK cells have two basic functions – cytotoxic, which determines the antiviral and anti-tumour response, and they are positioned for a key role in regulating autoimmune responses. Maturing, differentiating and their normal activity depends among other things on an autocrine/paracrine cytokine effect, including IL-2, IL-12, IL-15, IL-18, IFN-alpha and -gamma [16, 17]. The correct function and quantity of NK cells have a significant influence on the HCV infection's course. When NK cells are cultured with HCV replicon-containing hepatic cells, they have no direct cytolytic effect but release soluble factors suppressing HCV RNA expression. This effect is directly proportional to the number of added NK cells. IFN gamma is the main factor in this mechanism. The role of IFN-gamma in NK anti-HCV activity is supported by the following:

Ligation of CD81 on NK cells inhibits IFN-gamma production and results in decreased anti-HCV activity. In addition, the antibodies to IFN-gamma or IFN-gamma receptors abolish the anti-HCV activity of NK cell-conditioned media [18].

Bonawita and coworkers, while giving patients with chronic HCV infection IFN-alpha, observed a normalization of previously decreased NK cell count and a significant increase in their cytotoxic activity [19]. Similar results were achieved using IL-2 [20]. An increase in NK cell count and the concentration of IFN-gamma in IFN-alpha treatment was also observed by Nguyen. It was proven that patients who as a result of successful antiviral treatment have a normal NK cell count have a HCV reactivation much less often than those with a stable NK deficit [21].

The correct NK cell activity is controlled by a balance between activating and inhibitory signals, which work through a differential surface receptor matrix. Despite the few well-documented reports which confirm NK cell function defects, most viral infections are associated with proliferation and the NK cells' activity increases.

How to explain the deficit, or even their absence in the HCV-TP patient group? It seems that decreased number and activity of NK cells in a lingering HCV infection's course could be a result of their annihilation in the "apoptosis – mediated physiological depletion" mechanism [22]. In lingering viral infections a function defect of NK cells is reported more often than their deficit. It mostly manifests itself by disabling the cytolytic activity. NK function defect, in patients with an HCV infection, is explained by changes in the surface restraining CD94/NKG2A receptor's expression [16]. In an in vitro test the NK cells isolated from patients' blood had a lower ability to annihilate hepatoma cells and lower ability to produce IFN-gamma than NK cells which came from healthy donors. After blocking the receptor with anti-NKG2A antibodies, NK cells' cytotoxic activity rose, and an increase in IFN-gamma concentration as well as an increase in proliferation and activity of dendritic cells was observed [16, 23].

Besides the cytolytic function, NK cells have an important role in regulating autoimmune responses [24, 25]. Among other things they restrain proliferation and differentiation of B lymphocytes through IFN-gamma [26]. Many studies document decreased NK cell numbers or disabling of their function in the peripheral blood of patients with auto-immunological illnesses such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), Sjögren's syndrome, rheumatoid arthritis (RA), type I diabetes, Basedow-Graves' disease, Hashimoto's thyroiditis and ITP. There are described cases of SM, SLE, ITP progressions connected with simultaneous NK cell count drop [24, 27, 28]. Another argument for significant NK cell participation in autoimmune syndromes' pathogenesis is animal model testing.

Depletion of NK cells before infection with CVB3 rendered resistant strains of mice sensitive to the development of myocarditis accompanied by auto-antibodies against cardiac myosin as susceptible strains of mice [29].

As a result of NK cell depletion a rapid colitis progression [30], severe autoimmune myasthenia gravis with the presence of auto-antibodies against the acetylcholine receptor [25] in a mouse and autoimmune encephalomyelitis in rats [31] were observed.

Gathering reports, both our own and other authors', the role of NK cells in auto-immunological thrombocytopenia pathogenesis in HCV infection has to be underlined. It seems that decreasing their number can not only disturb the process of limiting viraemia, but also through proliferation and B lymphocyte activity's growth can participate in initiating production of auto-immunological antibodies, including those against platelets.

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