Importance of NK cells in predicting the effect of IFN-α (Roferon A) and thymus factor X (TFX) therapy in patients with chronic hepatitis C

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Abstract

Introduction: *In this study, percentage value and absolute number of NK cell were assessed in patients with chronic hepatitis C (CHC) before and after 3 and 6 months of treatment with interferon (IFN-α) and thymus factor X (TFX). We tried to find importance of NK cells to predict the therapy results. Moreover we evaluated the effect of the therapy on function and morphology of the liver.*

Material and Methods: *The study included 36 patients with CHC aged between 21-63 years (mean age 41. 36±10.25). Combined therapy with IFN-α 2a and a TFX was applied. Evaluation of percentage value and absolute number of NK cells was performed on peripheral blood cells using standard techniques for whole blood immunofluorescence labeling before therapy in 3rd and in 6th month of therapy. The patients were divided into two groups: group 1 patients with sustained viral response (SVR) (negative HCV-RNA in the serum 6 months after the end of the therapy), group 2 patients without SVR. Changes in NK cells were compared before and during the treatment in relation to viral response. To evaluate influence of the therapy on function and morphology of the liver we compared the results of liver biopsy and plasma clearance with 99m Tc- HEPIDA before and 12 months after the therapy. The results were analyzed statistically.*

Results: *SVR (absence of HCV-RNA 6 months after therapy – group 1) was gained in 10 from 36 (27.8%) patients. In the liver biopsy (one year after the end of therapy), statistically significant ($p<0.05$) decrease of necrotic and inflammatory changes intensity was found in the group 1. In group 1 percentage values of NK cells in 24th week were statistically higher in comparison with the values before the therapy ($p<0.05$). In 24th week of the therapy the statistically significant higher percentage value and absolute number ($p<0.05$) of the NK cells in group 1 in comparison with the values in group 2 (patients with no SVR) were found.*

Conclusions: *The obtained permanent viral response (in 27.8% patients) improved liver function and caused decrease of histopathological changes. Statistically significant increase in the percentage of NK cells in patients with SVR, after 24 weeks of IFN-α 2a and TFX compared with the result before the therapy was observed. The percentage and absolute number of NK cells were statistically higher in patients with permanent viral response, which is our contribution and may help to predict the course of the therapy.*

Key words: *IFN, TFX, NK cells, HCV.*

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Introduction

Chronic hepatitis C (CHC) is a slowly developing disease which may lead to cirrhosis or primary liver cancer. It is caused by Hepatitis C Virus (HCV) and because of being common it is one of the major hepatic problems.

Mechanisms of specific and natural immunity are responsible for fighting viral infections. Natural immunity consists of different genetically conditioned mechanisms which protect against infections. 1). Natural killer cells (NK cells) play an important role in this process. NK cells start to multiply and their cytotoxic activity increases under the influence of interferons, particularly their alpha (IFN-α) and beta (IFN-β) types [1]. In contrary to macrophages they are able to recognize and destroy selectively the infected cells. Thymus hormones increase NK cells potential by stimulating lymphocytes to produce many cytokines (i.e. IFN-α and IFN-γ, IL-2, IL-4). Hence it may be assumed that treatment with IFN-α and TFX may improve the therapy results. At present the accepted standard treatment is combination therapy using pegylated IFN-α and ribavirin. SVR using this therapy (negative HCV – RNA 6 months after the end of the therapy) was obtained in 50-60% patients [2, 3]. This therapy is not only accompanied by many side effects but also very expensive. Hence it cannot be used in all patients. So new, safer, more effective and cheaper methods of CHC treatment are being looked for. It also seems worth searching for favourable prognosis factors which may contribute to monitoring therapy results and decrease the cost of the therapy.

The aim of the paper was:

1. The evaluation of the therapy with interferon α 2a (Roferon A) and TFX (thymus factor X) in patients with CHC.
2. The evaluation of the therapy on function and morphology of the liver.
3. Importance of NK cells to predict the therapy results.

Material and Methods

Characteristics of the patients

The investigations were carried out on 36 adult patients (16 females and 20 males) aged 21-63 years (mean age 41.36±10.25) treated for CHC in the Clinic of Infectious Diseases, Medical Academy in Łódź, in 1997-1999. Before the investigations the following qualifying and excluding criteria for participation were used.

Criteria qualifying patients for the study

1. Chronic hepatitis confirmed by histopathological investigations before the therapy.
2. Histopathological changes at least G1S2.
3. ALT activity over 60 u/l in at least two investigations.
4. Presence of HCV antibodies in the serum.
5. HCV-RNA in the serum.
6. Good general condition of the patient.
7. Written consent for the treatment.

Criteria excluding patients from the study

1. Cirrhosis of the liver.
2. Coexisting HBV (HBsAg in the serum).
3. After splenectomy.
4. Alcoholism or drug addiction in the interview.
5. After immunosuppressive on antiviral therapy in the last 3 years.
6. Over 65 years of age.
7. Cancer, diabetes, unstable coronary heart disease, autoimmune diseases, mental diseases, pregnancy.

Treatment used

All patients were treated with combination of Interferon α 2a (Roferon A-Roche) and TFX (Jelfa – Jelenia Gora). Roferon A, a single dose 6MU, was given subcutaneously 3 times a week (Monday-Wednesday-Friday). TFX, a single dose 10 mg, was given intramuscularly twice a week (Tuesday-Thursday).

Forty eight week therapy was planned. The course was reduced to 24 weeks if, after this time, HCV-RNA was found in the serum of the patients. Hence 19 patients underwent 48-week treatment, while 17 patients underwent 24--week treatment.

Investigations

Basic laboratory tests

Basic laboratory tests of the peripheral blood: complete blood cell count, activity of the selected "hepatic" enzymes (ALT, AST, GGTP, ALP), bilirubin concentration, prothrombin index, were carried out in each patient before, every two weeks during the therapy and also 12 and 24 weeks after the treatment. These investigations were used to monitor the safety of the treatment.

Investigations of the viral condition

The following viral markers: HCV antibodies, HBsAg were determined once before the treatment. The investigations were done using ELISA method with Organotechnika reagents in COBAS and ROCHE apparatus.

Each patient was tested for HCV-RNA in the serum using RT-PCR method before the therapy, in 12th and 24th week of the therapy and 24 weeks after the therapy.

Patients with longer therapy (19 persons) were additionally tested in the 48th week of the therapy. Sensitivity of the HCV RNA detection was 100 copies/ml.

Immunological investigations

Evaluation of absolute numbers and percentage of lymphocyte subpopulation in the peripheral blood was done before the therapy and in the 12th week of the therapy. The

evaluation was also done in 35 patients in the 24th week of the therapy.

Two ml of blood from the ulnar vein was obtained to a Vacutainer tube (Becton Dickinson, San Jose, the USA) which contained heparin (10 U/ml). The blood was analyzed 2-4 h after collection.

Evaluation of NK cells was carried out on the peripheral blood cells using standard techniques for the whole blood immunofluorescence labeling. 100 µl of the blood was shaken and incubated at room temperature with proper amount of monoclonal antibodies. Erythrocytes were eliminated by adding lysogenic fluid (Becton Dickinson) to the tube, short incubation and rinsing. Then the samples were fixed with Cellfix liquid. A set of monoclonal antibodies (Becton Dickinson) $CD3^{-}$ $CD16^{+}$ $CD56^{+}$ (NK cells) was used. For evaluation flow cytometer FACS Calibur with argon laser 488 nm (Becton Dickinson) was used. SimulSET program was used for result analysis. The results were presented as the percentage of positive cells in the investigated sample or as absolute number (number of cells/µl) using morphological evaluation of the blood, which was being done simultaneously.

For immunological investigations a reference group of 120 persons, chosen according to age, described by Zeman et al. was used [4].

Histopathological investigations

Liver biopsy was done in all patients before the therapy but also in 30 patients in 12th month, after the therapy. Bioptate was taken using Menghini method in local anesthesia with disposable HEPAFIX sets produced by Braun. The evaluation of the infection grading and liver fibrosis staging were done according to Scheuer scale.

The evaluation of liver parenchyma function

Before the therapy liver plasma clearance with 99m Tc-HEPIDA was estimated in all patients. The investigation was repeated in 30 patients 12 months after the end of the therapy.

Criteria for dividing patients into groups

Patients who underwent the therapy were divided retrospectively into 2 groups:

Group 1:

- SVR

(negative HCV-RNA in the serum 6 months after the end of the therapy).

Group 2:

- no SVR

(positive HCV-RNA in the serum 6 months after the end of the therapy).

Statistical analysis

To assess differences in percentage values and in absolute number of NK cells of the peripheral blood between the CHC patients and the healthy controls and between group 1 and group 2 of patients, we used the Student's *t*-test for normally distributed variables and the Mann-Whitney U-test for variables which were not distributed normally. Differences in percentage values and in absolute number of NK cells in CHC patients before treatment and in the 3rd and 6th months of therapy were evaluated statistically by analysis of variance with post hoc comparisons. Statistical significance was defined as a value $p<0.05$.

Results

Evaluation of ALT normalization and HCV-RNA

Normalization of ALT activity was observed in 21 out of 36 patients (7 from group 1 and 14 from group 2) after 12 weeks of the therapy while negativization of HCV-RNA in 22 patients (10 from group 1 and 12 from group 2).

Normalization of ALT activity was observed in 15 out of 36 patients from group 1 and 8 from group 2 in the 24th week of the therapy while negativization of HCV-RNA in 19 patients (10 from group 1 and 9 from group 2).

Six months after the end of the therapy normalization of ALT activity was observed in 12 out of 36 patients (10 from group 1 and 2 from group 2) while negativization of HCV--RNA was found in 10 patients (all from group 1 according to the criteria of the group division).

Evaluation of the plasma clearance with 99mTc-Hepida

Mean value of plasma clearance with 99m Tc-Hepida both before the therapy and 12 months after the end of the therapy was decreased in each of the investigated groups compared with laboratory norms ($p<0.05$), the value of plasma clearance with 99m Tc-Hepida before the therapy and 12 months after the end of the therapy was not different in patients with no SVR.

Values of plasma clearance with 99m Tc-Hepida increased after the therapy for all the investigated patients and also for patients with permanent viral response($p<0.05$). The value of plasma clearance with 99m Tc-Hepida before the therapy and 12 months after the end of the therapy was not different in patients with no permanent viral response.

Histopathological investigation

According to score evaluation, no statistically significant data in necrotic-infectious changes and fibrosis in the liver biopsy were observed before and one year after the therapy.

In group 1 statistically significant decrease in necrotic--infectious changes in the liver biopsy was observed one year after the end of the therapy ($p<0.05$) while fibrosis did not change.

In group 2 no statistically significant changes were found both in the evaluation of necrotic – infectious changes and fibrosis in the liver biopsy before the therapy and one year after the therapy.

Results of NK cells investigations (tables 1 and 2)

Before the therapy the percentage of NK cells was significantly lower in all investigated patients compared with the control group ($p<0.05$). The investigated group did not show statistically significant changes in the percentage of these cells in the course of the therapy. However, statistically significant increase in the number of NK cells was observed in 24th week of the therapy compared with the results obtained in the 12th week of the therapy ($p<0.05$).

In group 1 the percentage of NK cells was significantly higher than in the control group in 24th week of the therapy ($p<0.05$). Moreover, in 24th week of the therapy the increase in the percentage of NK cells compared with the results obtained before the therapy was statistically significant ($p<0.05$).

In group 2 before the therapy the percentage of NK cells was significantly lower compared with the control group. No statistically significant changes were observed both in the percentage and absolute values. Moreover, the increase in the percentage and number of NK cells was statistically significant in group 1 compared with group 2 in 24th week of the therapy ($p<0.05$).

Discussion

The paper presents patients with CHC treated with combination of two immunomodulating drugs: thymus peptide (TFX) and interferon α 2a (Roferon A), which additionally shows antiviral activity. Favorable viral result was obtained in 10 out of 36 patients (27.8%). Our result is better, compared with the result obtained by the authors using only IFN-α monotherapy in Polish patients, often infected with genotype 1b HCV (about 84%), which is most resistant to the treatment [5]. In Cianciara study [6] the permanent response was observed only in 9.4% patients, however, the single doses of interferon were lower (3 MU 3 times a week) the therapy lasted 6 months and the total dose of the drug was 234 MU per patient. Niwicka-Michałowska [7] used 5 MU IFN-α 3 times a week for 24 weeks (360 MU/therapy) and obtained permanent viral response in 18.6% patients. In our study the patients were given 6 MU of the drug 3 times a week for 24 weeks and in case of negativization of HCV-RNA in 24th week of the therapy the treatment was prolonged to 48 weeks. Nineteen patients treated for 48 weeks were given maximal total dose of 864 MU/therapy while 17 patients were given 432 MU/therapy (6 MU 3 times a week for 24 weeks). The therapy was well tolerated by all patients but one who suffered from aggravated depression symptoms.

So far the results of only few research projects evaluating combination drug therapy with IFN-α and different thymus hormone: thymosin – α 1 (TA-1) in patients suffering from CHC have been published. However, most studies were carried out on a small group of patients. Sherman et al. [8] presented the largest group of patients treated with IFN-α and TA-1. They described the results of the therapy in 109 patients: 35 patients were given IFN-α and TA-1, 37 patients only IFN-α and 37 patients – placebo (double blind trial). The therapy lasted 6 months. Patients treated with IFN-α were given 3 MU of the drug 3 times a week. TA-1 was given 1.6 mg twice a week. Evaluation of biochemical (normalization of ALT activity) and viral response was done after the end of the therapy. Normalization of ALT and negativization of HCV-RNA was obtained in 37.1% patients treated with both drugs. In patients treated with only IFN-α the results were worse: 16.2% and 18.9%, respectively. Histopathological investigation showed improvement in liver

Table 1. Changes in percentage values of NK cells during therapy

NK cells (%)	All patients	Group 1	Group 2	Control group
before treatment n=36	14.72 ± 7.43^{I}	15.20 ± 9.43^{A}	14.54 ± 6.72^{II}	17.2±6.1
3 months of treatment n=36	15.61±6.65	17.30±10.55	14.96±4.50	
6 months of treatment n=35	17.84±6.77	$22.1\pm6.97^{I\,\#}$	15.91±5.86	

Mean and SD are shown.
Patients with CHC before treatment compared with control group: ^{I} – $p<0.05$.
Patients with CHC (group 2) before treatment compared with control group: ^{II} – $p<0.05$.
Patients with CHC (group 1) before treatment compared with the 6th month of therapy: ^{A} – $p<0.05$.
Group 1 compared with group 2 in 6th month of therapy: $^{\#}$ – $p<0.05$.

Table 2. Changes in absolute number of lymphocytes during therapy

NK cells/µl	All patients	Group 1	Group 2
before treatment n=36	357±246	358±258	357±246
3 months of treatment n=36	299 ± 159^{D}	315±219	293±134
6 months of treatment n=35	378±185	$482\pm200^{\#}$	330±160

Mean and SD are shown.
The 3rd month of therapy compared with the 6th month of therapy: ^{D} – $p<0.05$.
Group 1 compared with group 2 in 6th month of therapy: $^{\#}$ – $p<0.05$.

bioptate taken after the end of the therapy from patients treated with IFN-α and TA-1: the improvement was not observed in patients treated with only IFN-α or with placebo. Unfortunately, SVR (at least 6 months after the end of the therapy) was not investigated in this study. Even better results of IFN-α therapy were obtained by a group of Italian and American researchers [9]. They used IFN-α, 3 MU 3 times a week and TA-1 1 mg twice a week, for 12 months. Viral response to the therapy was evaluated after the therapy and 6 months later. Negativization of HCV-RNA was obtained in 73% and 40% of patients, respectively. These are very good results, however, the group of patients was small (15 patients).

Moscarella et al. [10] also evaluated the efficacy of combination of IFN-α, 3 MU 3 times a week and TA-1, 1.0 mg twice a week therapy; the control group were patients treated with only IFN-α. Each group consisted of 17 patients. Biochemical response was obtained in 11/17 (65%) and 5/17 (29%), respectively. It is not known if viral response was permanent. In this study we obtained favorable biochemical effects in 15/36 patients (41.7%) in 24th week of the therapy and viral response was observed in 19/36 (52.8%) in 24th week of the therapy. Normal ALT activity was observed in 12/36 (33.3%) and negativization of HCV--RNA in 10/36 (27.8%) 6 months after the end of the therapy. Four patients showed increased ALT activity although HCV-RNA in the serum was not observed in 24th week of the therapy. However, each patient with negative HCV--RNA in the serum showed normal ALT and AST 6 months after the end of the therapy. Our results of normalization of ALT activity and negativization of HCV-RNA in the serum at the end of the therapy were similar to the results presented by Sherman et al. [8]. Moscarella et al. [10] and Rasi et al. [9] presented significantly better results [10], however, their studies were carried out on smaller group of patients. Permanent viral response was evaluated in Rasi et al. study [9] and was significantly higher than the result obtained in our investigation (40% compared with 27.8%) The increase of the dose of IFN-α from 3 MU to 6 MU did not improve therapy results in our group of patients. It must be stressed that the researchers mentioned above used synthetic TA-1 while our patients were given partly standardized preparation – extract from calf thymus. [11].

In Andreone et al. [12] pilot study viral response was better in patients treated with IFN-α and TA-1 compared with patients treated only with IFN-α at the end of the therapy but it was similar in both groups 6 months after the end of the therapy.

Addition of TFX to standard treatment with pegylated IFN-α with ribavirin might improve current results. It is indicated by the results of introductory investigations using combination of TA-1, ribavirin and IFN-α [11].

Hepida clearance increased significantly 12 months after the therapy compared with the results before the therapy which may indicate improvement in liver function.

The most important investigation in evaluating progress of the disease, predicting its course and also qualifying patients with CHC for the therapy is histopathological evaluation of liver bioptate. Development of fibrosis is the consequence of infection. Progressing fibrosis leads to changes in liver structure and development of cirrhosis.

Most papers present improvement in histopathological picture of the liver after IFN-α therapy in patients with CHC [13, 14]. However, favorable histopathological changes do not refer to all treated patients. Sherman et al. [8] observed higher histopathological improvement in liver bioptate in patients after IFN-α and TA-1 therapy compared with those after only IFN-α therapy.

The improvement may depend on the therapy duration, kind of drugs used and also the time of control biopsy.

We observed statistically significant decrease in necrotic-infectious changes in control biopsy in group 1 (with permanent viral response) and no improvement in group 2. We did not observe any statistically significant improvement in fibrosis in either of the investigated groups. Other authors also observed histopathological improvement in patients with permanent viral response [13].

IFN, complement, NK cells, macrophages, granulocytes are most important elements of natural response involved in fighting viral infection.

Virus infected cells and some neoplastic cells are the aim of NK cell cytotoxic attack. IL-2, IFN-α, IFN-β, IL-12, IL-15 and IL-18 are the main factors stimulating NK cells. The increase in the number of these cells is important in fighting viral infections and neoplastic processes. Hence, theoretically, IFN-α and TFX therapy should increase the number of NK cells. Chan et al. [15] found that the number of NK cells was the same both in patients with CHC and healthy persons. Similar results were presented by other authors [16, 17].

Shupper et al. [16] observed an increase in the number of NK cells in patients with CHC while Appasamy et al. [18] found the increase in the number of activated NK cells. Some authors obtained results similar to ours and found lower percentage and number of NK cells in patients with CHC [19, 20]. These differences may be due to different methods of evaluation of lymphocyte subpopulations in the peripheral blood and also to different age of the patients. Some authors also evaluated the influence of IFN-alpha therapy on NK cells behavior in patients with CHC. Jirillo et al. [19] observed the increase of initially lower percentage of NK cells in the course of the therapy in patients with normalization of ALT activity. Stachowski et al. [20] found higher increase in the number of NK cells, in the course of the therapy, in patients with HCV elimination. Woźniakowska--Gęsicka et al. [17] observed a similar influence of IFN-α on this subpopulation of cells. After 6 months of IFN-α therapy in a group of children the percentage of NK cells increased compared with a group of healthy children and a group of infected children but not treated. The percentage was higher in treated children with elimination of infection compared

with treated children with no elimination of infection. However, Russo-Mancuso et al. [21] observed the increase in the number of NK cells in patients with elimination of infection and decrease in patients in which the multiplication of the virus was not inhibited despite IFN-α therapy. Panasiuk [22] found increased percentage of NK cells in 3 rd month and then decrease in 6th month of the therapy, however, he did not divide patients into groups according to the obtained results. These papers stress the increase of percentage and number of cells in this subpopulation especially in patients who respond well to the therapy. According to Appasam [18] exogenous interferon may change the balance between favorable (antiviral activity) and unfavorable (liver damage) effect of NK cells activity.

It seems that behavior of NK cells may play an important role in predicting the effects of the therapy in patients with CHC.

The results of the paper and cited literature suggest that supplementation of the CHC therapy with thymus hormones may prove profitable.

Conclusions

1. The obtained permanent viral response (in 27.8% of patients) improved liver function and caused decrease of histopathological changes.

2. Statistically significant increase in the percentage of NK cells in patients with permanent viral response after 24 weeks of IFN-α 2a and TFX compared with the result before the therapy was observed. The percentage and absolute number of cells NK cells was statistically higher in patients with sustained viral response, which is our contribution and may help to predict the course of the therapy.

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