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• *BRIEF REPORTS* •

Effect of interferon alpha2b plus ribavirin treatment on selected growth factors in respect to inflammation and fibrosis in chronic hepatitis C

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Abstract

AIM: Growth factors (GF) that participate in regeneration and apoptosis have an important role in chronic liver diseases. We analyzed serum GF concentration during antiviral treatment and correlated it with morphological liver failure in chronic hepatitis C.

METHODS: The levels of GF were determined in sera by ELISA method in 0, 16, 32 and 48 wk of therapy in 40 patients treated with IFNα2b (9 MU sc/wk) and RBV (1.2 g/d) and in 25 healthy subjects. Blind liver biopsies were done before treatment with histological grading and staging examination.

RESULTS: The hepatocyte growth factor (HGF) and epidermal growth factor (EGF) were markedly elevated prior the treatment and decreased during the therapy, although they did not reach the normal level. In non-responding (NR) patients, HGF and EGF were higher than that in responders (R), however differences were not significant. Before the treatment thrombopoietin (TPO) level was significantly lower in R than in NR (P<0.03). Platelet-derived growth factor (PDGF) concentration was lower in chronic hepatitis C than in healthy subjects and decreased during the treatment. A significant positive correlation was observed between inflammatory activity in the liver tissue and the concentration of HGF (in R: r = 0.4, in NR: r = 0.5), TPO (R: r = 0.6), and a significant negative correlation between this activity and EGF (R: r = -0.6) and PDGF (R: r = -0.5). Serum HGF concentration was higher in more advanced fibrosis (R: r = 0.5, P<0.05; NR: r = 0.4, P<0.03).

CONCLUSION: The decrease in PDGF can be an effective prognostic marker of the treatment and HCV elimination. Decreasing HGF, EGF, and PDGF can influence the inhibition of inflammatory and fibrotic processes in the liver during the antiviral treatment.

INTRODUCTION

Viral hepatitis C (HCV) infection is a frequent reason of chronic hepatitis. Immunological phenomena in HCV infection are not clear and mechanisms of natural elimination of HCV and effective therapy have not been established so far. Liver morphologic changes caused by chronic HCV infection can lead to inflammation and fibrosis[1]. Persistent HCV infection can be the cause of secretion disorders as for proteins and factors essential for the liver and other organ functioning[2,3]. The expression of specific molecules on infected hepatocytes induces the immune system activation. Natural mechanisms are not usually capable of fighting the infection. Factors of cellular environment responsible for virus persistence and replication have not been known yet. HCV virus affects, directly and indirectly, the regeneration and apoptosis of infected cells[4,5]. The administration of exogenous interferon alpha alone or in combination with nucleoside analogue increases possibilities of HCV elimination[6-8]. Some growth factors (GF, hepatocyte growth factor - HGF, epidermal growth factor - EGF) and cytokines (IL-6) protect the liver against cytotoxic cell reactions. GF are produced by parenchymal (thrombopoietin - TPO) and non-parenchymal liver cells (HGF, EGF, and platelet-derived growth factor - PDGF) during liver infection. They have synergic and sometimes antagonist effect on immunological and inflammatory processes in the liver[9-12]. The knowledge of liver regeneration mechanisms can be helpful in the development of new treatment essential for induction of repair processes.

We analyzed serum growth factor levels in the course of treatment with interferon alpha 2b (IFNα2b) and ribavirin (RBV) in patients with chronic hepatitis C. We examined the relationship between growth factor concentrations and morphological changes in the liver, viral clearance, and biochemical parameters during therapy.

MATERIALS AND METHODS

Patients

The studies were conducted in the group of 40 patients (16 women and 24 men), aged 38.5±9.0 year, with chronic hepatitis C. Chronic infection with HCV was demonstrated by the presence of anti-HCV antibodies (ELISA method) for at least 6 mo and the presence of viral replication. Serum HCV-RNA was confirmed in all patients by RT-nested PCR in whole blood. Biochemical and hematological examinations monitoring the treatment effect were performed in 0, 16, 32 and 48 wk of therapy. The liver biopsies by means of the Hepafix System (Braun, Melsungen, Germany) and liver histopathological assessment were performed. Periportal and intralobular activities as well as fibrosis stage were analyzed using point assessment according to Scheuer's classification[13]. The microscopic examination of liver biopsy was presented in Table 1. The patients fulfilled all indicatory criteria for antiviral treatment and were given interferon alpha 2b (Intron A, Schering Plough, USA) in the dose of 3 MU sc thrics a week for 48 wk and ribavirin (Rebetron, Schering Plough) in the dose of 1.2 g daily for 48 wk. The patients were not given preparations affecting the immune system in the course of treatment. The study was continued in patients who had negative HCV-RNA in peripheral blood after 24 and 48 wk of the treatment. The qualitative HCV-RNA examinations carried out 24 wk after completing the treatment was the basis for the division of the patients into sustained viral responders (HCV-RNA negative) (R) and non-responders (HCV-RNA positive) (NR). The protocol of study was approved by the local bioethical committee.

The control group consisted of 25 healthy subjects (10 women, 15 men), aged 29.4±10.2 years, with no liver damage diagnosed.

Growth factors

Venous blood was collected in the morning using plastic tubes, before the treatment, and after 16, 32 wk and in the 48th wk of the treatment. Blood was centrifuged at 1 000 r/min within 60 min of collection and obtained sera were stored at -76 ℃. GF were assayed in duplicate with the quantitative sandwich enzyme immunoassay (EIA) technique.

Table 1 Microscopic examination of liver biopsy in patients with chronic hepatitis C according to Scheuer classification scale[13]

Histological liver examination Grading (stage)	Responders	Non-responders
Portal activity of inflammation: 1/2/3/4 (*n*)	3/7/16/0	1/5/12/0
Lobular activity of inflammation: 1/2/3/4 (*n*)	12/9/1/0	6/11/1/0
Staging (stage): 1/2/3/4 (*n*)	19/3/0/0	9/7/2/0

Hepatocyte growth factor Murine anti-HGF monoclonal antibodies were precoated as a solid phase onto a microplate (human HGF, R&D System, Oxon, UK).

Epidermal growth factor Murine anti-EGF monoclonal antibodies were precoated as a solid phase onto a microplate (human EGF, R&D System, Oxon, UK).

Total platelet-derived growth factor Murine anti-PDGF-BB monoclonal antibodies as a solid phase precoated onto a microplate (human PDGF-AB, R&D System, Oxon, UK).

Thrombopoietin Murine anti-TPO monoclonal antibodies as a solid phase precoated onto a microplate (human TPO, R&D System, Oxon, UK). Standard and samples were added into the wells and every growth factor was bound by immobilized antibodies. After washing away any unbound substances, polyclonal antibodies against HGF, EGF, PDGF-AA or monoclonal antibodies against TPO conjugated to horseradish peroxidase were added to the wells. After washing, substrate solution with stabilized hydrogen peroxide and tetramethylbenzidine were added to each well. The reaction was stopped by 1 mol/L sulfuric acid. Optical density was determined by microtitre plate photometer Stat Fax® (Alab, Poland) at 450 nm, corrected by subtraction of readings at 540 nm. The values of GF in a sample were established by interpolation from a standard curve calculated with standard samples added to kits by manufacturer.

Statistical analysis

The results were presented as mean±SD. Statistical analysis was performed using Student's *t* test for pairs. Parameter correlation was analyzed using Pearson's parametric correlation test and Spearman's non-parametric test. Statistically significant differences were considered for $P<0.05$.

RESULTS

Combined treatment of IFNα2b+RBV caused HCV elimination in 22 subjects (55%) out of 40 patients. The decrease of alanine aminotranferase (ALT) activity was observed in all patients during the treatment (Table 2). Suppression of bone marrow function was demonstrated by temporal decrease of platelet, leukocyte, and erythrocyte counts (non-significant statistically).

TPO values increased during the treatment and reached statistically significant level in the 32 wk (Table 3). However, it did not correlate with decreasing blood platelets count. Before the treatment, virological responders had TPO baseline concentration on the controls' level and it was significantly higher than that in non-responders ($P<0.03$) (Table 4). The treatment caused TPO values to increase in the responders (R) and decrease in NR. Serum TPO concentration revealed positive correlation with inflammation activity in the liver tissues (periportal $r = 0.5$, $P<0.01$,

Table 2 Biochemical and hematological parameters during IFNα2b with RBV therapy in chronic hepatitis C

Time of the treatment	Alanine aminotransferase (U/L)	Prothrombin index (%)	Platelets (1×10^3/μL)	Erythrocytes (1×10^6/μL)	Leukocytes (1×10^3/μL)
0	106±50	95±9.6	208±34	4.6±0.5	6.3±2.1
16 wk	24.4±16.7	97±6.4	198±46	4.0±0.5	5.1±2.3
32 wk	19.6±13.2	99±9.2	202±63	3.9±0.6	4.6±1.5
48 wk	35±67	100±7.4	193±62	4.1±0.6	4.9±1.9

intralobular $r = 0.6$, $P<0.004$) or fibrosis ($r = 0.4$, $P<0.04$) (Table 5).

In chronic hepatitis C, serum HGF concentration was higher before antiviral treatment than in controls and after the beginning of the treatment, HGF values diminished (Table 1). There were no statistically significant differences between HGF concentration in R *vs* NR before and during the treatment. Significant correlation between serum HGF concentration and stage of liver fibrosis (R: $r = 0.5$, $P<0.05$; NR: $r = 0.4$, $P<0.03$) and intralobular grading of inflammatory activity (R: $r = 0.4$, $P<0.05$; NR: $r = 0.5$, $P<0.03$) were observed. In treated patients, a significant positive correlation between serum HGF and EGF concentrations ($r = 0.2$, $P<0.04$) was also noticed.

EGF concentration in chronic hepatitis C is significantly higher than that in healthy individuals. In the 0-16 wk of IFNα2b+RBV treatment, we noted significant decrease in serum EGF concentrations, whose values were elevated in the following weeks (Table 3). In non-responders, EGF concentrations before the treatment were higher than that in responders (Table 4). There was a significant negative correlation between serum EGF values and histological inflammatory activity (periportal $r = -0.6$, $P<0.03$, intralobular $r = -0.5$, $P<0.01$) in responders. There was no statistically significant relationship between EGF concentration and fibrosis progression in the liver (Table 5). EGF concentration correlated significantly with HGF ($r = 0.2$, $P<0.04$) in NR and PDGF ($r = 0.5$, $P<0.04$) concentration in R during the treatment.

PDGF concentration in chronic hepatitis C was lower than that in the control group. Before the treatment, PDGF concentrations in R were higher than that in NR. Antiviral treatment IFNα2b+RBV caused PDGF drop in responders and elevation in NR as compared to initial values (Table 4). We observed a significant negative correlation between PDGF concentrations and periportal ($r = -0.5$, $P<0.01$) and intralobular ($r = -0.5$, $P<0.02$) inflammatory activity in R. Despite therapy effectiveness we did not observe any relationship of PDGF in respect to stage of liver fibrosis (Table 5).

The study showed no significant correlation between aminotransferase, bilirubin values, blood cell count, and growth factor concentrations determined during the therapy.

Table 3 Level of GF in chronic hepatitis C during IFNα2b with RBV therapy (mean±SD)

	HGF (pg/mL)	EGF (pg/mL)	TPO (pg/mL)	PDGF (pg/mL)
Healthy	748±91	12.9±3.3	47.6±16.5	473.2±145
0 (I)	983±227	18.4±7.1[a]	55.5±17.2	387±116
16 wk (II)	752±277	12.7±6.3	71±56.3	334±84.8[a]
32 wk (III)	783±297	13.9±5.2	67.5±18.3[a]	342±84.5[a]
48 wk (IV)	770±278	15.8±5.7	62.5±28.1	330±138[a]
P value	NS	I:II $P<0.01$	NS	I:II $P<0.02$
		I:III $P<0.01$		I:III $P<0.03$
		II:IV $P<0.04$		

[a]$P<0.05$ Student's *t* test.

Table 4 Serum concentrations of GF in virological responders and non-responders, Student's *t* test (mean±SD)

Growth factor	Responders		Non-responders		*P*	
	0 wk	48 wk	0 wk	48 wk	0 wk	48 wk
HGF (pg/mL)	873±210	792±198	919±257	742±260	0.6	0.7
EGF (pg/mL)	16.8±5.8	16.1±6.2	20.4±8.4	15.5±4.9	0.2	0.8
TPO (pg/mL)	48.4±14	68.8±13	64.2±17.1	54.8±12	0.03	0.2
PDGF (pg/mL)	425±111	292±180	340±111	377±98	0.1	0.1

DISCUSSION

Combined administration of interferon alpha and ribavirin leads to HCV elimination and inhibition of inflammatory reaction and liver fibrosis in some patients[8,14]. In our study, we observed a relationship between inflammatory activity or fibrosis stage and serum GF concentrations in chronic hepatitis. Thrombopoietin concentrations increased whereas HGF, EGF and platelet-derived factor decreased during IFNα2b+RBV therapy. There were differences in GF concentrations in respect to virological responders or non-responders.

In hepatitis, the sources of PDGF, beside activated platelets, are macrophages and Kupffer cells[15,16]. The essence of biological significance of this connection is the stimulation of fibrogenesis and mitogenesis of Ito cells in the liver[17]. PDGF along with transforming growth factor β_1 (TGFβ_1) are the most powerful inductors of liver fibrosis. As we have demonstrated recently there is a significant correlation between plasma TGFβ_1 and the degree of liver insufficiency in patients with liver cirrhosis[18]. According to Mannaioni *et al*[15], as liver damage intensifies, PDGF is elevated in the serum and PDGF-R in inflammatory infiltrations along vessels scattered in the liver connective tissues and on proliferative Ito cells. Chronic hepatitis reveals a high correlation between PDGF-R expression, activity, and morphological change progress and collagen deposition[16]. During IFNα2b with RBV administration we observed the decrease in serum PDGF concentration. It is a favorable effect of the treatment which can cause the reduction of Ito cell activation and inhibition of fibrosis. It is in accordance with other authors' observations that showed inhibitory IFN alpha influence on liver fibrosis[8,19]. Excess of PDGF inhibits thrombocytopoiesis in chronic liver diseases. Therefore, we

Table 5 Correlation of GF in the course of IFNα2b with RBV therapy with grade activity of inflammation and fibrosis in the liver in responders (R) and non-responders (NR). Histological classification according to Scheuer[13]

Growth factor		Inflammation activity, grading		Fibrosis hepatitis, staging
		Periportal	Intralobular	
HGF	R	NR	$r = 0.4$, $P<0.05$	$r = 0.5$, $P<0.05$
	NS	NS	$r = 0.5$, $P<0.03$	$r = 0.4$, $P<0.03$
EGF	R	$r=-0.6$, $P<0.003$	$r = -0.5$, $P<0.01$	NS
	NR	NS	NS	NS
TPO	R	$r=0.5$, $P<0.01$	$r = 0.6$, $P<0.004$	$r = 0.5$, $P<0.01$
	NR	NS	NS	$r = 0.4$, $P<0.04$
PDGF	R	$r=-0.5$, $P<0.01$	$r = -0.5$, $P<0.02$	NS
	NR	NS	NS	NS

r - statistical correlation, Spearman's non-parametrical test, $P<0.05$ statistically significant, NS – no statistical significance.

did not observe thrombocytopenia in patients treated with IFN with RBV.

The liver cells are the main place of TPO production, which is the crucial stimulating factor in megakariocytopoiesis and thrombocytopoiesis. Its amount is strictly related to the degree of liver cell efficiency. The decrease in hepatocyte functionality and intensification of liver fibrosis affect TPO production causing its decrease[20]. Moreover, HGF influences TPO production in the liver[11]. TPO concentration is markedly higher in chronic viral hepatitis than in other liver diseases[21]. However, we did not observe any correlation between serum TPO concentration and mRNA TPO in liver tissues in various liver diseases[21]. Our studies showed high values of thrombopoietin in patients with chronic hepatitis C. During IFNα2b+RBV treatment, TPO concentrations increased, but it did not correlate with platelet decrease. Higher TPO concentrations (statistically significant) were observed in non-responders that were lowered in the course of therapy. In chronic hepatitis C, R showed a positive correlation between TPO values and the grade of histological inflammation, whereas in NR the correlation was positive for TPO and the stage of liver fibrosis.

HGF is a pleiotropic growth factor of mitogenic, motogenic, and morphogenic properties, and inhibits certain neoplasm growth[22]. HGF is produced in high quantities in acute infections, such as sepsis, influenza, pneumonia, and acute hepatitis[23]. Immunohistochemical studies in chronic hepatitis reveal higher percentage of Kupffer cells and Ito containing HGF than in liver cirrhosis[24]. HGF stimulates the production of acute phase proteins (alpha 2-macroglobulin and albumin) of hepatocytes in primary culture. However, Shiota *et al*[25], studies did not show the relationship between HGF and acute phase proteins, which correlated with liver damage. Their further studies showed the positive correlation between HGF and total bilirubin, aspartate aminotransferase and the negative correlation with prothrombin time and albumin concentration[26]. We did not observe any association between these parameters and HGF concentration in chronic hepatitis C before and during the therapy. HGF concentration increases together with the degree of liver insufficiency. The highest concentration occurs in cirrhotic patients with Child-Pugh class C[26]. In chronic hepatitis, HGF was significantly related to histological activity index score and fibrosis stage[26,27]. Yamagami *et al*[27], in studies on various stages of liver damage showed the highest HGF values in acute hepatitis, lower in liver cirrhosis and chronic hepatitis. Intrahepatic HGF expression shown in focal necrosis is related to serum level of HGF[27]. Our studies showed high HGF values in chronic hepatitis C that correlated positively with histological grading of inflammatory intralobular activity and the stage of liver fibrosis independent on IFNα2b+RBV treatment efficacy. During the treatment we observed a positive relationship between HGF and EGF concentrations in NR patients. Serum HGF values were lower in responders than in patients not responding to the treatment. During the treatment HGF values decreased and were comparable to those of healthy subjects. So we suggest that determination of HGF concentration in serum can be helpful in defining inflammatory activity and fibrosis in the liver. EGF, beside HGF, causes the increase in hepatocytes proliferation. HGF and EGF administered to the environment of stem bone marrow cells caused the occurrence of features typical for hepatocytes. It might give hope for cell culture for liver transplant[28]. EGF and hepatotoxic preparations (thioacetamide, carbon tetrachloride) given to animals decrease organ damage and morbidity as compared to the control group without EGF[29]. Komuves *et al*[30], showed that EGF transcription was highly elevated in cirrhotic liver (regenerative nodules, bile duct epithelia) as compared to low expression in normal liver.

We revealed significantly higher EGF values in chronic hepatitis C than in healthy subjects. Serum EGF values decreased in the first period of the treatment, however during the therapy it slowly increased. Higher EGF concentration occurred in non-responders during the therapy. The positive correlation between EGF and the grade of inflammatory activity in NR and HGF and PDGF concentrations in the course of IFNα2b with RBV treatment in both groups were observed.

By analyzing the dynamics of GF in responders and non-responders we observed that PDGF decrease might be the predictive marker of the effective treatment and HCV elimination. Higher TPO concentration can be a protective mechanism against thrombocytopenia during IFNα2b with RBV treatment. It seems that decreasing values in HGF, EGF, and PDGF reflect stabilization of inflammatory and fibrosis processes in the liver as an effect of the therapy.

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