Pathogenesis of hepatitis C virus (HCV) infection: role of genetic, immunologic and environmental factors in HCV-related liver disease and in symptomfree HCV carriers.

Hepatitis C virus (HCV) infection is a major global health problem: 170 million subjects are suffering from chronic HCV infection worldwide, of whom five million in Europe and about 70.000 in Hungary. About 80 % of patients with acute HCV infection go on to chronic hepatitis, from which later cirrhosis and/or hepatocellular carcinoma may develop, representing the most frequent indication of liver transplantation.

The *outcome of HCV infection is highly variable* from spontaneous viral clearance to symptomfree HCV carrier state with persistently normal alanine aminotransferase (ALT), or to chronic hepatitis and cirrhosis. Some cases in HCV infection show rapid progression – while others remain „healthy" carriers. It is not completely understood what may be the exact cause of the different outcome, but it is suggested, that partly *direct viral cytopathy*, partly host-related *genetically determined immune mechanisms* as well as *environmental factors* are responsible for the HCV-induced tissue injury.

Since a strong NK and T-cell response is essential for the clearance of HCV, **genetic factors,** as well as the type of the **immune response** are of particular importance in the pathogenesis. In addition, **environmental** factors may also be capable of modifying the host's response and the outcome of HCV infection.

We investigated some of these factors, such as haemochromatosis gene mutations, genetic polymorphism of CTLA4 and angiotensin converting enzyme (ACE) genes, features of NK and T cell functions and viral coinfections, in different forms of chronic HCV infection, that is in symptomfree HCV carriers with persistently normal alanine amino transferase (ALT) and in patients with chronic hepatitis C, treated with Peg-IFN + Ribavirin (RBV) therapy..

We wanted to elucidate the potential causes determining the differences between the above mentioned groups of HCV infected individuals, as well as between rapid, early or slow responders and non-responders to antiviral therapy.

Genetic studies

The natural history of HCV infection remains controversial. Chronic hepatitis C is typically characterised by slowly progressive hepatic fibrosis. However it is recognised, that some patients *do not progress, while others rapidly develop cirrhosis*. It is difficult to assess an *individual's risk* of progressive liver disease. Such prognostic uncertainty and variation in the response to the infective agent, suggest a genetic component to the outcome of HCV infection. Thus, among factors, that influence the rate of progression, the *genetic predisposition may be crucial.*

HFE gene mutations

We have shown, that the *allele frequency* of *haemochromatosis gene mutations (C282Y and H63D)* in patients with chronic HCV hepatitis *did not differ from that of the normal Hungarian population*.

CTLA4 polymorphism

CTLA4 is a T cell surface molecule, an inhibitory T cell receptor, expressed on activated and regulatoy T lymphocytes. Its binding partners, CD80 and CD86 are expressed on professional antigen presenting cells. Binding CD28 molecule on antigen

presenting cell, CTLA4 is a *negative regulator* of T cell activation, playing a role in maintenance of peripheral self tolerance. *The lack of CTLA 4* - or its certain *single nucleotide polymorphisms* (SNPs) such as -318>T in the promoter region and +49A>G in the first exon, resulting in CTLA4 functional abnormality, - impairs the control of T cell response, predisposes to loss of self tolerance, and can lead to excessive (uncontrolled) immune reactions, even autoimmune diseases, e.g. immune thyreoiditis.

Since *CTLA4 polymorphisms* can contribute to development of *excessive immune reaction*s, it is tempting to speculate, that the same genetic variations *may protect from infections* in other individuals. Thus, it can be hypothetised, that the same polymorphisms may be *predictive of a favourable outcome* of treatment in HCV patients On the other hand, it may be related to immune *thyroiditis,* the known extrahepatic complication of HCV infection as well as side effect of interferon therapy. We have investigated the role of SNPs -318 and +49 in patients with different types of HCV infection, in symptomfree „healthy" HCV carrier state, in patients with active chronic hepatitis C, responders and non-responders to antiviral tratment. We wanted to evaluate the influence of these polymorphisms on the outcome of IFN-RBV based antiviral therapy and the immune mediated side effects of the treatment. Up to now, *we have found no differences concerning CTLA4 polymorphisms between "healthy" virus carriers and patients with active HCV hepatitis, as well as between responders and non-responders to antiviral treatment.*

Angiotensin converting enzyme (ACE) gene polymorphism

Since progressive **fibrosis** is a key element of HCV induced liver disease and the r*enin-angiotensin system (RAS) and angiotensin II in particular, play an importan role in the regulation of fibrosis in several organs,* the relationship of hepatic fibrosis and RAS sysem have been suggested.

Angiotensin II (AGII) is known as a principal effector molecule of *renin-angiotensin system, and it also* mediates production of **TGF-β**1. AG II induces expression of **TGF-β**1 through ANG II type 1 receptor. **TGF-β**1 increases collagen deposition with accumulation of extracellular matrix. by controlling transcriptional regulation of fibrillar collagens. Through this mechanism ANG II has been implicated in the development of fibrosis in renal and cardiac diseases, and probably similar mechanisms are at work in liver cirrhosis as well. Recently the presence of angiotensin receptors was shown even on hepatic stellate cells.

Many functional polymorphisms in the genes of the RAS have been described and postulated to contribute to the inter-individual variation in development of several renal an cardiovascular diseases, but yet not in liver cirrhosis.

First we studied the ACE gene **I/D** polymorphisms in patients with chronic hepatitis C, and assessed their effect on the course of the HCV-disease, in PEG-IFN + RBV treated patients. It was found, that among *HCV-patients who responded with sustained virological response (SVR) to the antiviral therapy, the ACE D/D mutant occurred in 51.8% vs in non-responders of 23.1%, suggesting a beneficial effec of ACE deletion on the outcome of anti-HCV treatment.* Further studies are warranted to explore the background of this finding.

Immunological studies, fibrosis and oxidative stress

Natural killer cell (NK) activity, perforin expression and expression of CD81 molecule.

Impaired **NK cell activity** has been proposed as a mechanism contributing to viral persistence in hepatitis C virus (HCV) infection. We have shown that in patients with hepatitis C, the

natural killer (NK) activity of the *peripheral blood mononuclear cells (PBMCs) was significantly lower as compared* not only to normal control, but to *symptomfree virus carrier*s and to those, who responded to antiviral (PEG-IFN + ribavirin, RBV) therapy with complete sustained viral clearance. PEG-IFN + RBV treatment resulted in elevation of NK activity of PBMCs even after one week of therapy and remined elevated during the entire treatment period.

It was also demonstrated, that the frequency of ***perforin-positive cytotoxic CD3+ T*** cells was lower in chronic hepatitis C patients as compared to healthy virus carries. On the antiviral treatment, the number of perforin-positive T cells increased, and this rise was sustained only in those who were able to clear the virus.

We have shown the ***overexpression of CD81 molecule***, a surface co-receptor for HCV, on peripheral blood NK and B cells of patients with active hepatitis C. At the same time, in healthy HCV carriers the expression of CD81 was decreased as compared to patients with active hepatitis C. PEG-IFN + RBV treatment resulted in decreased CD81 expression in patients with sustained virological response (SVR)

Natural killer (NK) cell function, activator receptors, regulatory T cells and TGFbeta1

Since *NKG2D* as an important **activator** receptor of NK cells, has a pivotal position in both innate and adaptive immunity and as it is expressed not only on NK cells, but also on CD8+ T cells, we analyzed whether decreased NK activity corresponds to a dysregulated expression of NKG2D on NK and CD8+ T cells. As *regulatory T (Treg) cells* are an important source of TGFbeta1, correlations between the percentage of Treg cells, plasma TGFbeta1 levels and NKG2D expression of NK and T cells were also studied.

The percentage of peripheral CD4+CD25high+Treg cells, NKG2D+ NK and T cells were determined by flow cytometry in patients with chronic hepatitis C, in sustained virological responders (SVR) previously treated with interferon (IFN) and in healthy controls.

Results: In patients with chronic HCV hepatitis the activating receptor NKG2D expression was significantly downregulated both on NK and on T cells compared to healthy controls. This impaired expression of NKG2D was associated with increased proportion of CD4+CD25high+ Treg cells and increased TGFbeta1 levels compared to control group. TGFbeta1 levels inversely correlated with the surface expression of NKG2D on NK cells. In contrast, the percentage of Treg cells, TGFbeta1 levels and the expression of NKG2D in sustained virological responders was comparable to that of healthy controls.

Conclusion: Our data present the first evidence that TGFbeta1 - secreted by regulatory T cells - is responsible for impaired NK cell function via down-regulating NKG2D activating receptor in chronic HCV hepatitis. Thus, TGFbeta antagonism or soluble NKG2D ligands may provide the basis of a novel antiviral therapy to improve the function of NK and T cells.

Phenotypes and cytokine production of peripheral blood mononuclear cells

Since less is known of the immunologic basis of rapid virological response (RVR) to IFN + RBV therapy in chronic hepatitis C, we compared the phenotypes and cytokine production of peripheral blood mononuclear cells of rapid, slow and non-responders before treatment initiation. Fifty patients with chronic HCV hepatitis before and at 1, 3 months of PEG-IFN+ RBV treatment were studied. The percentage of CD4, CD8, CD56, CD19, Treg, CD14 cells and TNFalpha, IL-2, IL-6, IFNgamma, IL-4, IL-10 production of LPS stimulated monocytes and PMA+ionomycine stimulated peripheral blood lymphocytes were determined by Flow Cytometry. Baseline, LPS-induced TLR4 activation of the monocytes resulted in significantly higher Th1 type cytokine (TNFa:and IL6) production in rapid responders, compared to slow responders and also to non-responders. Rapid HCV RNA clearance was also associated with decreased Th2 type cytokine production of lymphocytes. IL-4 and IL-10

production of lymphocytes were significantly higher in slow responders compared to rapid responders. TNFalpha production of monocytes was predictive for rapid virological response. While PEG-IFN and RBV treatment increased IL-2, IFNgamma, TNFalpha production in slow responders, it had no effect on non-responders. An elevated TNFa and IL-6 production of TLR4 stimulated monocytes, increased IFNg and diminished IL-4 and IL-10 production of peripheral blood lymphocytes were associated with rapid virological response.
Conclusion: Pretreatment TLR4 activation of the monocytes induced significantly higher Th1 cytokine production in rapid responders compared to patients with lack of RVR, suggesting that modulation of TLR activity and cytokine production in non-responders may play important role in new therapeutic strategies. Determination of cytokine production may help identify patients more likely to respond to antiviral therapy as well as provide a rationale for the further design and use of immunotherapeutic approches.

Fibrosis markers

Since in the outcome of chronic hepatitis C virus (HCV) infection the progression of **hepatic fibrosis** is essential, and IFN treatment is supposed to inhibit fibrogenesis, we wanted to compare changes in three non-invasive **fibrosis markers** in chronic HCV hepatitis. Plasma levels of *TGF-beta1* and *hyaluronic acid (HA)* were determined by ELISA, *procollagen-III*-peptide (P-III-P) levels by RIA in symptomfree HCV carriers and in patients with chronic hepatitis C before the antiviral treatment and 1, 3, 6 and 12 months thereafter. Twenty two patients became responders (R), 27 patients were non-responders (NR). Correlation between TGF-beta1, HA, P-III-P levels and the histological activity and the fibrosis score in liver biopsy was evaluated. Pretreatment plasma TGF-beta1, HA and P-III-P levels were significantly ($p<0,01$) increased in both responder and non-responder HCV-patients compared to controls. HA levels correlated with fibrosis score, TGF-beta1 with histological activity index. PEG-IFN + RBV treatment decreased both TGF-beta1 and HA levels, not only in responders but also in non-responders. The reduction of fibrosis marker levels was more considerable after 6 months of antiviral therapy, and remained sustained even 6 months after the treatment. PEG-IFN plus Ribavirin treatment decreased TGF-beta1 and HA levels independently of virological response. In symptomfree HCV carriers levels of fibrosis markers did not differ from the normal control.
Conclusion: that antiviral treatment with PEG-IFN plus RBV in chronic hepatitis C may have antifibrotic effect even in virological non-responders.

Oxidative stress

Effects of silymarin supplementation in PEG-IFN + RBV treated chronic hepatitis C.
Since oxidative stress may play a pathogenetic role in chronic hepatitis C and the sustained virological response to antiviral therapy is limited in HCV1 genotype infection, a double blind study was performed in PEG-IFN + RBV treated HCV1 patiens, to assess the efficacy of the supplementation with an antioxidant flavonoid, silymarin.

Thirty-two naive HCV1 positive patients with biopsy proven chronic hepatitis C, to be treated with PEG-IFN + RBV have been randomized: group A): 16 patients were given the antiviral therapy for 6-12 months plus placebo for the first 3 months, group B): 16 patients were treated with pegylated interferon + ribavirin for 6-12 months plus silymarin 2x166 mg/day was given for 3 months. Serum alanine aminotransferase (ALT) and HCV-RNA levels, as well as parameters of oxidative stress such as plasma or red blood cell hemolysate malondiadehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase and myeloperoxidase (MPX) were determined at 0, 1, 3, 6

and 12 months during the treatment. sustained virological response as undetectable serum HCV RNA was evaluated after 24 weeks of the end of therapy. In the silymarin group a more rapid decrease in MDA level, as well as a marked decrease in SOD and an increase in MPX activity at month 12 were found.
Conclusion: Although **s**ilymarin supportation to antiviral therapy improved oxidative stress, it was not able to affect favourably the sustained virological response. These contradictory findings may be related to randomization bias, as patients in the study group B have more negative predictors of virological response: they were older with higher fibrosis score and even with more severe pretreatment baseline oxidative stress. Regarding the recently published *in vitro* experiments with silybinin on HCV replication as well as the newest convincing clinical observations, we suggested futher studies with more than three times higher doses silymarin in controlled trials to asses the effect of silimarin supportation in anti-HCV-treated partients.

Environmental factor: Co-infection with SEN virus in HCV-patients

It has been suggested, that **co-infections with hepatotropic viruses** may play a role in the different outcomes of HCV infection. Yet, the implications of these co-infections in the clinical course are unclear, and it should also be elucidate, whether the response type to anti-HCV treatment is influenced or not by their simultaneous presence.

We determined the prevalence of ***SENV DNA D/H*** in patients with chronic HCV infection, and evaluated the effect of SENV infection on the PEG-IFN + RBV induced sustained virological response (SVR). In addition, the effect of the anti-HCV therapy on SENV elimination was assessed. Three groups of HCV patients have been studied: group A): 19 individuals with HCV-RNA positivity and persistently normal serum ALT level, group B): 48 patients with chronic active hepatitis C, and group C): 18 pts with HCV cirrhosis. Thirty-four healthy individuals served as control. Serum HCV-RNA was measured by Cobas Amplicor HCV Monitor 2.2 Roche, presence and quantity of SENV-D and SENV-H strains was determined by Real-time PCR using Taqman probes. In the healthy controls, SENV-D prevalence was 8.8% and SENV-H 38.2%, in group A) 42.1% and 31.5%), in group B) 43.7% and 66.6%, in group C) 27.7% and 66.6%, respectively. Of 19 HCV patients with SVR to PEG-IFN + RBV, 42.1% were SENV-D carrier and 63.1% were SENV-H positive, while in 29 non-responders 44.8% and 68,9%, respectively. *SVR* occurred among SENV D/H DNA positive patients in 37.8%, in SENV-D positive cases 33%, in SENV D/H negative patients in 45% of cases, respectively. SENV-D co-infection seems to be a negative predictor of virological response in PEG-IFN + RBV treated chronic hepatitis C. The *anti-HCV treatment* resulted in SENV D/H elimination in 40.5% of cases. In the group of non-responders to PEG-IFN + RBV therapy, half of SENV-H positive patients showed an elevation of SENV-H DNA titer during the course of hepatitis C.
Conclusion: The prevalence of SENV infection is higher in patients with chronic active C hepatitis/cirrhosis compared to healthy controls. The efficacy of anti-HCV treatment was unfavourably affected by the presence of SENV-D, but not SENV-H. SENV seemed to be sensitive to PEG-IFN + RBV therapy.

Summary

1. Neither haemochromatosis gene mutations, nor CTLA4 polymorphisms influence the activity of HCV-related liver disease, however, ACE gene deletion has a favourable effect on the outcome of anti-HCV treatment in chronic hepatitis C.

2. Overexpression of CD81 molecule on NK and B cells, as well as transforming growth factor beta1 (TGFbeta1) secreted by regulatory T cells, and downregulated NKG2D activating receptor on cytotoxic effector cells, may play a privotal role in the impaired cell mediated immunity in active HCV infection

3. Plasma TGFbeta1, hyalurinic acid and procollagene-III-peptide levels – as markers of fibrogenesis - are elevated in active hepatitis C patients, but not symptomfree carriers. Interferon + ribavirin therapy may inhibit fibrogenesis independently of virological response in chronic HCV hepatitis.

4. SEN virus co-infection, - as an additive environmetal factor - frequently occuring in HCV patients, can decrease the effect of anti-HCV treatment.