Abschlussbericht Teilprojekt 12.4

Projekttitel: Mechanismen der Interferon-Resistenz des Hepatitis C-Virus und die Bedeutung viraler Faktoren

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I. Short description

Aims of the project

Many HCV strains, first of all of genotype 1, are resistant to treatment with interferon. The mechanisms of such a resistance are still unclear although some indications on the involvement of viral factors are presented.

The first aim of the proposed project was to study a possible contribution of 3‘NCR of HCV genome to interferon resistance. The project assumed comparative analysis of viral sequences corresponding to 3‘NCR from IFN sensitive and resistant HCV strains. This study was directed at the identification of particular mutations or sequence motifs in 3‘NCR that might be associated with the IFN resistance of some of HCV variants.

The second aim was to investigate the possible contribution of the NS5A region to the interferon resistance of some HCV strains. The study assumed the construction of the series of hybrid HCV subgenomic replicons in which the NS5A sequence was swapped by corresponding fragments from interferon- sensitive and -resistant HCV isolates of the HCV.

Background

Combined therapy with interferon–alpha (IFN-α) and ribavirin nowadays remains the only available option for treatment of patients with chronic hepatitis C. Unfortunately, many patients, especially those infected with HCV type 1 strains, often demonstrate no sustained virological and biochemical response to the therapy (Pawlotsky et al., 1999). The exact mechanisms by which HCV circumvents the antiviral effect of IFN remain unknown. The one working hypothesis assumes that gene product(s) of the HCV-resistant variant disrupt IFN-induced anti-viral responses at different levels and that these virus - host cell interactions are associated with IFN resistance. According to this hypothesis the course of IFN therapy may lead to the selection of one or several resistant HCV variants bearing particular mutations in a definite region of the viral genome. Thus, the major current avenue of research has its aim in the establishment of the genetic determinants of the HCV genome that might contribute to interferon resistance. One such a candidate could be 3‘NCR, involved in a regulation of viral replication. The HCV 3‘NCR is capable of binding a number of cell proteins, and some of the 3‘NCR-cell protein interactions may

represent a mechanism by which HCV evades the effects of the interferon-inducible enzymes protein kinase R and 2‘-5‘ oligoadenylate synthetases. Identification of mutation in the 3‘NCR and their functional analysis could have contributed to a clarification of the mechanisms of interferon resistance.

The data, available by the time the current project has been initiated, suggested the involvement of viral factors, in particular, of the viral non-structural protein NS5A, in the mechanisms of IFN-resistance (Enomoto et al., 1995; Pawlotsky et al., 1999; Rispeter et al., 1999; He and Katze, 2002). A number of clinical observations suggested a correlation between the NS5A sequence, and, in particular of the so-called IFN sensitivity determining region (ISDR) and IFN response. Series of in vitro experiments with different types of cells, overexpressing NS5A protein, provided the first experimental data on the modulation of the IFN-response by this viral protein (He and Katze, 2002), however, such systems might have not completely modeled the situation occurring in natural infection of human hepatocytes. A possibility to study the role of NS5A under “more physiological conditions” appeared after development of the HCV subgenomic replicon model (Lohmann et al., 1999; Blight et al., 2000). Several laboratories demonstrated that replication of HCV replicons in Huh7 cells was sensitive to exogenous IFN-α (Blight et al., 2000; Frese et al., 2001; Guo et al., 2001), and the observed effects seemed to be independent of the type of NS5A. These results did not confirm the previous clinical and laboratory observations, thus adding some confusion with regard to the role of NS5A in the mechanisms of IFN-resistance. Considering all this it looked worthwhile to initiate the investigations of the involvement of NS5A in regulation of the response to interferon in vitro by using the series of the HCV subgenomic replicons; in which NS5A region was derived from IFN-resistant and IFN-sensitive HCV isolates.

Materials and methods

The serum samples from two groups of patients were used to obtain materials for virological investigations: (1) pre-selected and already carefully investigated (Wiese et al., 2000) cohort of women infected with the same virus strain AD78 during the outbreak of hepatitis C in Germany in 1978 after introduction of contaminated anti-D immunoglobulin; (2) patients chronically infected with HCV type 1.

The following plasmids were used throughout the study: (1) The subgenomic HCV replicon; based on the consensus sequence of the Con1 strain of HCV (plasmid pFK-I_{389}/NS3-3´) (Lohmann et al., 1999); (2) The variant of this replicon (plasmid pFK-I_{389}/NS3-3´/ET), containing the adaptive mutations in NS3 (E1202G) and T1280I) and NS4Bb (K1846T), (Lohmann et al., 2003); (3) cDNA clone with a consensus sequence of AD78 strain of HCV (Rispeter et al., 1997).

Standard virological and molecular biological methods were used throughout the study.

Cooperation within the HepNet

All investigations performed in the framework of the project were a result of a close collaboration between several researchers, participants of the HepNet: Prof. E. Screier, Berlin; Prof. M. Wiese, Leipzig, Dr. V. Lohmann and Prof. R .Bartenschlager, Heidelberg, Prof. G. Gerken, Essen. Such collaboration made it possible to select strictly defined patients groups, like, for example, two subgroups of AD78 infected patients, IFN-responders and nonresponders, and to obtain serum samples as a starting material for the subsequent experiments. Besides that, in the process of investigations there was a regular exchange of such materials, as recombinant plasmids and transfected and untransfected cells. This shearing of materials significantly reduced the workload of different groups and speed up their investigations

II. Detailed description

Scientific results

In general, the whole study was done in full accordance with the program and the plan envisaged in the initial project proposals. According to the plan, the first step of the project comprised a comparative analysis of viral sequences corresponding to the 3'-UTR from IFN sensitive and resistant HCV 1b strains obtained from chronic hepatitis C patients subjected to IFN-ribavirin therapy. The fragments corresponding

to a fragment of 3‘-UTR were amplified by RT-PCR from sera of 30 HCV type 1-infected patients (15 responders and 15 non-responders), cloned in pCR2.1-TOPO vector and the resulting 2 to 5 clones were sequenced in two directions. This work has been done in collaboration with Dr. E. Schreier, Berlin; Dr. T. Berg, Berlin, and Dr. G. Gerken, Essen. The technical procedure included the reverse transcription at elevated temperature and the PCR under conditions, which allowed for a more accurate and reproducible amplification of the molecules with expressed secondary structures. The sequences of the „variable region“ and of poly-U/UC tract were subjected to a further analysis. The sequence of the „variable“ region, which is quite different in isolates belonging to different HCV types, was found to be very conservative among the investigated viral isolates belonging to the same type. The few exchanges observed were located mostly in stretches not involved in base pairing and, therefore, may not influence the putative secondary structure of this region. In contrast, the poly-U/UC region demonstrated high level of heterogeneity both with regard to the length and the composition. Very often the amplification of the 3‘-UTR resulted in appearance not of the one but of two distinct fragments of different length. Cloning and sequencing of these fragments demonstrated the variability of the length of poly U/UC tract in these molecules. Thus, for example, the 3‘UTR amplificates from one sample comprised of molecules containing poly U/UC stretch of 78, 70, 68, and 45 nucleotides. Within poly U stretches of several C appeared at intervals 1 to 8 U residues. These motifs formed a core consensus sequence UCUU for polypyrimidine tract-binding protein (PTB). Occasionally, the poly U/UC stretch included small numbers of A or G. In general, the sequence pattern of the poly-U/UC tract was heterogeneous among individuals. Within the same individual such heterogeneity was less evident and characteristic patterns for each sample or even for each patient were observed. Future studies should answer the question on the biological significance of the differences in the sequence patterns within the poly-U/UC tract, in particular, of the differences in the number of putative PTB motifs. Comparison of the sequences of the 3‘-UTR fragments amplified from sera of IFN responders and non-responders did not reveal the presence of any sequence pattern or motif, associated with the response to the IFN therapy. We were also unable to identify any particular difference in the sequence pattern in „variable“ region or poly-U/UC tract in viral isolates obtained before and after the IFN therapy in non-responders. Several of the revealed variants of the 3’-UTR sequences were cloned

into the subgenomic replicon. The resulting hybrid replicons were tested in cell culture. The data obtained with naturally occurring variants of 3'-UTR confirmed the results reported earlier for synthetic 3'-UTR (Frieze and Bartenschlager, 2002), namely, that the length of the poly-U/UC tract influence the replicating potentials of HCV subgenomic replicon. Several hybrid replicon variants, in which poly-U/UC tracts with different number of potential PTB binding sites were included, were constructed. The testing of these constructs in Huh7 cells is underway. Preliminary data suggest that differences in the composition of the poly U/UC tract (number of putative PTB motifs) might influence the replication potentials of the subgenomic RNA molecule.

The second part of the project comprised the efforts directed at the construction of the subgenomic HCV replicon based on the consensus sequence of HCV AD78 strain. The AD78 strain of HCV caused the unique outbreak of hepatitis C in Germany in 1978 when a number of women were infected due to inoculation with contaminated anti-D globulin. In the preliminary studies of our group the complete genome of AD78 was amplified and the cDNA clone with a consensus sequence was constructed. In the second stage of the current project, the RNA, corresponding to this consensus sequence, was assessed for infectivity. The plasmid encoding the complete HCV genome was subjected to RNA transcription in vitro and the resulting transcript was used for intrahepatic inoculation of chimpanzees, which was previously infected with HCV 1b and cleared the infection without anti-HCV seroconversion (this work has been done in collaboration with Dr. J. Liang, NIH, USA). The inoculated animal was followed for 10 weeks. Weekly serum samples were tested for HCV RNA and anti-HCV as well as in T cell proliferation assay with core, NS3, NS4, and NS5 antigens at several time points. There was neither evidence of a new infection, nor an anamnaestic response of T cells. At the next stage, the fragments of the AD78 consensus sequence corresponding to different genes were installed into the functionally active subgenomic HCV replicon (this work has been done in collaboration with Dr. V.Lohmann, Heidelberg). Some of these chimeras, in particular, those with fragments of AD78 NS4A, NS4B, NS5A, and NS5B, turned out to be viable in Huh7 cells (Fig 1). The ongoing efforts are directed at the enlargement of the fragment derived from AD78 genome in the backbone of viable Con1 replicon obtained by Dr. V. Lohmann and Dr. R. Bartenschlager. In

particular, site-directed mutagenesis of possibly lethal mutations in the NS3 region of AD78 sequence was performed and the analysis of the resulting clones is underway. One of the changes tested was the mutation of the residue 470 in domain II of the NS3 helicase (R470M), which was fount to be a critical determinant in cell culture adaptation of at least one 1b (HCV-BK) and one 1a (H77) strains of HCV (Grobler et al., 2003). The introduction of this mutation, however, did not confer cell culture replication ability to the inactive chimeric replicon containing AD78 NS3 sequence. (Fig.1, indicated in bold). Analysis of other amino acid substitution that might have such a deleterious effect on replication is underway.

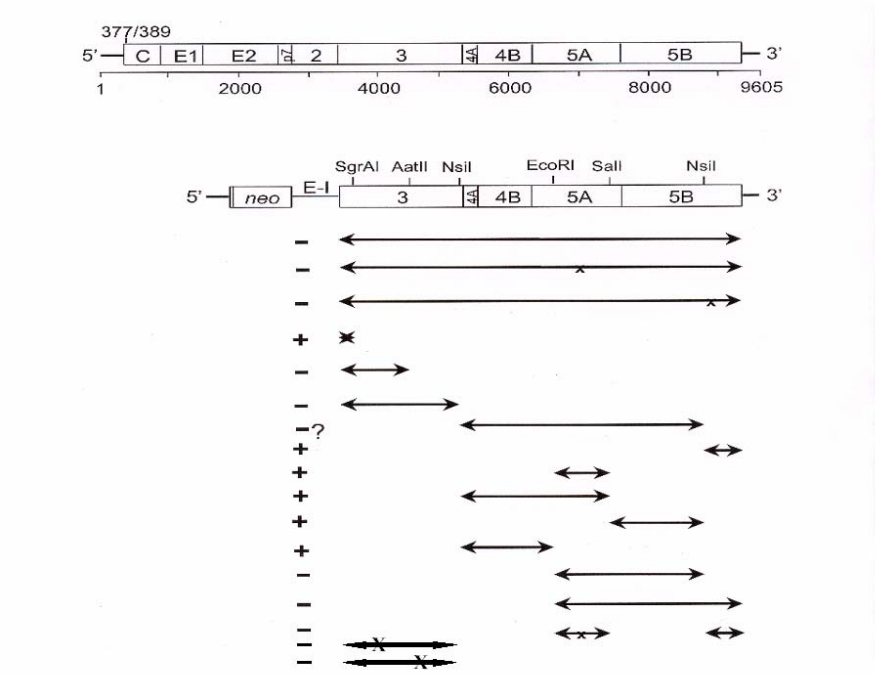


Fig.1. Construction of chimeric Con1-AD78 subgenomic replicons and their ability to replicate in Huh7 cells. Fragments with arrows indicate the fragments of AD78 swapped into the backbone of Con1 replicon. X – mutations introduced by site-directed mutagenesis. Replication competence of the chimeric clones indicated by + and -.

One of the chimeric constructs that demonstrated an ability to replicate in Huh7 cells was the Con1 replicon, in which NS5A sequence was derived from the consensus AD78 clone. This observation provided a basis for the series of experiments directed at the assessment of a possible role of NS5A in mechanisms of HCV resistance to interferon. As a source of the materials the serum samples from 4

IFN-responder and 4 IFN-nonresponder patients infected with AD78 strain were used (this work was done in collaboration with Dr. Wiese). DNA fragments corresponding to the NS5A region were amplified from sera of these patients and subjected to direct sequencing and the resulting deduced amino acid sequences, corresponding to the predominant virus type, were aligned with the sequence of the corresponding fragment from Con1 HCV isolate. The sequences of eight HCV AD78 derived isolates, including four
IFN-resistant ones (NR 1-4) and four IFN-sensitive isolates (R 1-4) differed from the Con1 sequence in at least 21 amino acid positions. Most of these substitutions, however, did not occur within the interferon sensitivity determining region (ISDR). Analysis of the amino acid variation of eight AD78-derived sequences did not reveal a particular mutation or a motif, associated with the IFN-resistant phenotype

The amplified NS5A fragments from eight AD78 isolates were additionally cloned and sequenced and the clones, containing the predominant variants of sequences were used for the construction of a series of hybrid Con1/AD78 subgenomic replicons, in which the original NS5A sequence of Con1-based replicon (Lohmann *et al*., 1999) was swapped by corresponding fragments from IFN-sensitive and -resistant isolates of HCV AD78 strain (Fig. 2).

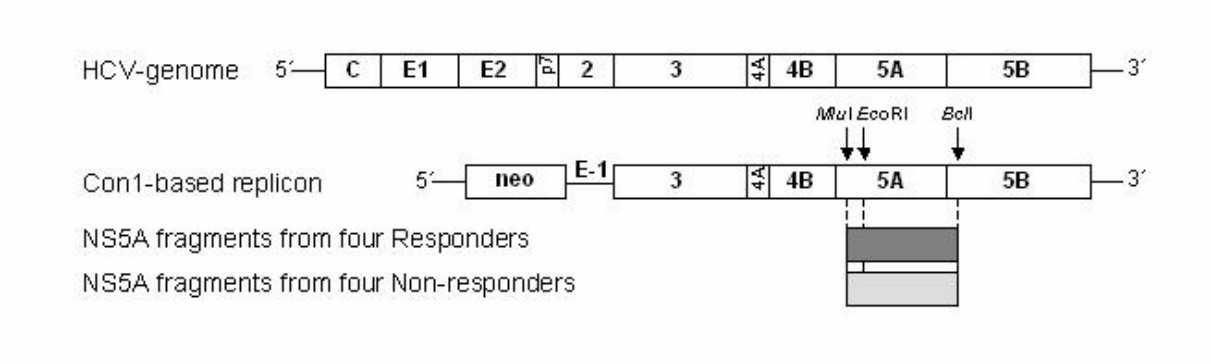
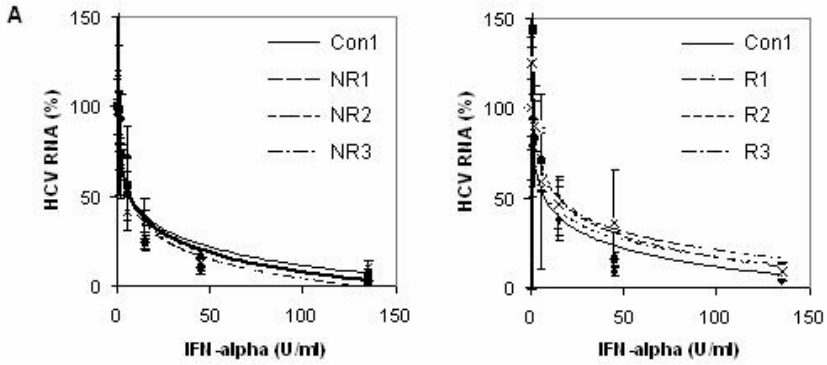


Fig. 2. Construction of hybrid HCV subgenomic replicons. A schematic presentation of the HCV genome organization is given at the top. The structure of the selectable HCV replicon and the inserted fragments of NS5A are shown below. The original NS5A sequence of the Con1-based replicon (plasmid pFK-I_{389}/NS3-3´/ET ; Lohmann et al., 2003*)* was swapped by corresponding fragments from IFN-sensitive and -resistant isolates of HCV AD78 strain. Arrows indicate the position of the restriction sites used for insertion of NS5A fragments into the original pFK-I_{389}/NS3-3´/ET plasmid.

The RNAs, transcribed in vitro from the prepared chimeric constructs, were used for transfection of Huh7 cells with a subsequent selection of G418-resistant cell clones. With the hybrid Con1/AD78 constructs, obtained from six patients, we were able to establish G418-resistant cell clones (R 1-3 and NR 1-3). Multiple attempts to transfect cells with the constructs bearing NS5A region from patients R4 and NR4 were unsuccessful. The Northern blot analysis of the total RNA from the established six cell clones demonstrated the presence of subgenomic HCV RNA molecules. Expression of NS5A protein in these cells was confirmed by Western blot. Quantification of the HCV RNA in these cell clones revealed a slight variability of the replicon copy number from $2{,}4x10^6$ to $1{,}2x10^7$ RNA copies per microgram of total RNA at different cell passages. The NS5A sequences of all chimeric replicons remained stable and similar to the original predominant sequences over a 5-month culture period of the current study.

The established clones of the Huh7 cells bearing the six chimeric replicons were used to assess the sensitivity of HCV replication to interferon. The transfected cells were treated with different concentrations of IFN-α. After 48 hours of incubation, total cell RNA was extracted and the number of replicon molecules was determined by a quantitative real-time RT-PCR (Fig. 3a). Dose dependent inhibition of HCV replication was observed and a 50% inhibitory concentration (IC_{50}) ^{for} each of six clones was determined (Fig. 3b). Repetitive experiments did not reveal significant differences in sensitivity of HCV RNA replication to interferon neither between cell clones, bearing replicons with NS5A sequences from IFN-responders and non-responders, nor between these clones and control cells bearing the original Con-1-based replicon.



B

Replicon	PC	R1	R2	R3	NR1	NR2	NR3
IC_{50}(U/ml)	8,2 ± 2,7	6,3 ± 2,9	7,8 ± 4,1	17,5 ± 8,0	6,5 ± 2,5	9,5 ± 4,8	15 ±1,3

Fig. 3. Inhibitory effect of IFN-α on HCV RNA levels in cells bearing the chimeric Con1/AD78 replicons. (A) Huh7 cells transfected with Con1 replicon (positive control, PC) or hybrid Con1/AD78 constructs, containing NS5A sequences of patients isolates (NR1 to NR3 and R1 to R3), were treated with 0; 0,55; 1,67; 5; 15; 45 and 135 U/ml IFN-α for 48 h, and assayed for the replicon copy numbers by quantitative RT-PCR. The HCV RNA levels of untreated control cells were compared with those found in cells treated with IFN-α, and the percentage of the remaining RNA was plotted against the IFN-α concentrations. The bars represent the standard deviation of triplicate experiments. (B) 50% inhibitory concentrations (IC_{50}) of IFN-α for chimeric Con1/AD78 replicons and for the original Con1 replicon (mean± SD).

The aim of the current study was to investigate a possible contribution of the NS5A region to the HCV replication resistance to interferon. The methodical approach used for this purpose provided several advantages. First, the use of a HCV subgenomic replicon system allows to study the role of NS5A in context of other HCV proteins, which is of special importance as many HCV proteins are known to function predominantly in complex with each other within the host cell (Penin et al., 2004). Second, the NS5A fragments were inserted into the backbone of Con1 replicon that had already been adapted for replication in Huh7 cells (Lohmann et al.,

2003). We assumed that such a replicon would tolerate the insertion of at least several native variants of NS5A. Indeed, the validity of this approach was demonstrated by the fact that we were able to obtain replication-competent replicons with six of eight constructs tested. Third, In our approach the HCV Con1 replicon was used as a backbone for swapping the NS5A fragment from IFN-resistant and non-resistant HCV isolates, which were obtained from patients infected with the same HCV AD78 strain in a single source outbreak (Dittmann et al., 1991; Wiese et al., 2000). This provided a unique opportunity to study and compare the sensitivity to interferon of HCV replicon molecules in which NS5A fragments from related but not identical variants of the same virus strain were used. Finally, one might have expected that the propagation of the cells, bearing the chimeric HCV subgenomic molecules, would lead to appearance of adaptive mutations in the NS5A. Such mutations could influence the sensitivity of these replicons to interferon. Fortunately, the NS5A sequences of all these six replicons were unchanged and identical to the original predominant sequences of natural HCV isolates, and remained stable for the whole period of our series of experiments (about 5 months). In this respect, our chimeric constructs behaved like a recently described Con1-derived subgenomic replicon K2040, which remained stable and unchanged over a 16-month period of cultivation in Huh7 cells (Sumpter et al., 2004). Using this experimental approach we tried to find a difference in response to IFN-α among the six of chimeric Con1/AD78 replicons. No such difference was registered however between replicons containing NS5A fragments from IFN-resistant and –sensitive isolates. Even more so, all replicons tested, including the original Con1 molecule, demonstrated very similar sensitivity to IFN-α (Fig. 4). Thus, our data provide no evidence that the NS5A protein contributes to the resistance of HCV replication to IFN-α.

Despite these results, we believe that the efforts directed at the establishment of the role of NS5A in the resistance of HCV to interferon should be continued. Thus, we cannot exclude that the Huh7 cells due to their specific characteristics do not provide the adequate biochemical environment for realization of all biological potentials of HCV gene products. From this point of view, it would be logical to extend the current study and to use cell lines other than Huh7 to analyze the sensitivity to interferon of HCV replicons, bearing different variants of NS5A.

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Publications

(1) Viazov S, Ross RS, Roggendorf M (2002) Hepatitis C virus persistence: role of viral factors in escape from the innate immune surveillance. Update in Hepatology, 10-27.
(2) aus dem Siepen M, Viazov S, Wiese M, Lohmann V, Roggendorf M. NS5A does not contribute to the resistance of HCV replication to interferon-alpha in cell culture (Submitted)
(3) Viazov S, Schreier E, Berg T, Roggendorf M. Heterogeneity of the 3'NTR of HCV and sensitivity to interferon (manuscript in preparation)

The obtained results were presented at the following international conferences:

- Annual Meeting of the "Gesellschaft für Virologie", Tübingen, 2004. A. aus dem Siepen, S.Viazov, M.Wiese, V.Lohmann, M. Roggendorf „Subgenomic HCV replicon as a tool for the study of the role of NS5A in interferon resistance of HCV"
- 11th International symposium on hepatitis C virus and related viruses, Heidelberg, 2004. A. aus dem Siepen, S.Viazov, M.Wiese, V.Lohmann, M. Roggendorf. „Non-structural 5a protein does not contribute to interferon resistance of HCV".

Abstract:

In cells infected with mammalian viruses antiviral responses exerted through a complex multilevel network of cytokines and transcription factors play an essential role in elimination of these viruses. During coevolution with their respective hosts many viruses, including hepatitis C virus (HCV), developed ways to circumvent host antiviral responses. The mechanisms by which HCV escapes the immune surveillance and establish a persistent infection are still not very well understood. This review focuses on the interactions of HCV with cell macromolecules and discusses the possible strategies used by HCV to evade the innate immunity.