Interferon therapy of hepatitis C: Molecular insights into success and failure

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Summary

20 years have passed since the discovery of the hepatitis C virus (HCV), and yet therapeutic options remain limited. Current standard treatment of chronic hepatitis C (CHC) consists of pegylated interferon alpha (pegIFNα) and ribavirin, and leads to a sustained virological response in approximately half of treated patients. Understanding non-responsiveness to pegIFNα, by analysing the molecular mechanisms underlying treatment failure, is important for future therapeutic improvements. In the following review the current status of knowledge on the crosstalk between HCV and IFNs, as well as on the molecular events occurring in liver tissue of HCV-infected patients in response to pegIFNα, is discussed. Furthermore, the review focuses on the prospect of developing a prognostic test that might direct treatment to those patients who will benefit from it. The outlook on novel therapeutics, including small molecule inhibitors of HCV proteins and immune modulators, is broadened by a glance at the exciting field of micro-RNAs that are likely to be implicated in viral replication and pathogenesis of CHC, thus representing a new therapeutic target.

Key words: Hepatitis C Virus; IFN-stimulated genes; STAT1 activation; liver biopsy; micro-RNA 122

Natural history and current treatment of HCV infection

Hepatitis C virus (HCV) infection, a major cause of liver cirrhosis and liver cancer, represents an enormous global health problem affecting 130–170 million people worldwide [1]. Since a protective vaccine against HCV does not yet exist and the therapeutic options remain limited, the number of patients presenting with long-term complications from this infection, and therefore also the number of patients in need for liver transplant, is expected to further increase in the next 20 years [2].

HCV was first identified in 1989 by screening serum from an infected chimpanzee with human serum from a non-A non-B post-transfusion hepatitis patient [3]. Before the development of an assay for HCV antibody detection as a screening measure [4], HCV was mainly transmitted through transfusions of blood products. Parenteral infec-
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Complete EVR (cEVR): Negative HCV-RNA at week 12 of treatment

End of treatment non-response: Detectable serum HCV-RNA at the end of treatment

End of treatment response (EoTR): No detectable serum HCV-RNA at the end of treatment

Sustained virological response (SVR): Undetectable HCV-RNA 6 months after EoTR

Relapse: Detectable HCV-RNA after having achieved EoTR

Definitions of treatment response.

Table 1

| Rapid virological response (RVR): | Negative HCV-RNA at week 4 of treatment |
| Early virological response (EVR): | HCV-RNA reduction >2 log_{10} after 12 weeks |
| Complete EVR (cEVR): | Negative HCV-RNA at week 12 of treatment |
| Primary non-response (PNR): | Less than 2 log_{10} decrease in viral titer after 12 weeks |
| End of treatment response (EoTR): | No detectable serum HCV-RNA at the end of treatment |

IFNα is crucial for the treatment of HCV infection and will continue to be indispensable also in the era of novel antiviral compounds that are currently evaluated in clinical studies (discussed in the chapter “The future of anti-HCV therapy”).
Antiviral effects of type I interferons and how HCV counteracts them

The IFN family of cytokines exerts antiviral, immunoregulatory and antiproliferative effects on target cells. 13 subtypes of IFNα and a single IFNβ isoform belong to type I IFNs in humans [21]. Type I IFNs are produced in response to pathogens as part of the innate immune system. RNA viruses such as HCVs are recognised in the infected cell by two pathways, one involving Toll-like receptor 3 (TLR3) and the other involving cytosolic RIG-I like helicases [22–24]. Activation of these pathways results in the induction of type I IFNs. Type II and III IFNs will not be discussed in this review. All type I IFNs bind to the same cell surface receptor, the IFNα receptor (IFNAR) consisting of two chains, and induce intracellular signalling through the Jak-STAT pathway [25] (fig. 1).

IFNs inhibit replication of numerous viruses, and are beneficial in the treatment of multiple sclerosis and malignancies including cutaneous melanoma, hairy cell leukaemia and renal-cell carcinoma [21]. The important role of IFNα in antiviral responses is based on direct antiviral actions through transcriptional activation of hundreds of IFN-stimulated genes (ISGs). Various ISGs with potent antiviral efficacies have been identified to date. Induction of these ISG-encoded proteins and their related pathways can lead to a block in viral transcription, degradation of viral RNA, inhibition of translation or interference with various steps of viral replication [26]. Protein kinase R (PKR), 2′–5′ oligoadenylate synthetase (OAS) and Mx proteins are among the best-characterised IFN-induced antiviral effectors. For example, the protein phosphatase 2A, finally resulting in ent degradation of STAT1 in Huh7 cells expressing HCV core protein [40] and proteasome-dependent degradation of STAT1 in Huh7 cells expressing HCV proteins [41]. Epidemiological studies show that the remaining 20–30% of patients overcome acute HCV infection, suggesting that innate and/or adaptive immune responses are indeed capable of controlling the outcome of HCV infection. Evidently, HCV cannot block IFN signalling proteins interfere with replication of negative-stranded RNA viruses such as influenza [28]. Important immuno-modulatory effects of IFNα are mediated through activation of natural killer cells and cytotoxic T lymphocytes, which recognise infected cells and attempt to clear them [29].

The exact mechanisms by which endogenous or therapeutically applied IFNα exerts its effects against HCV remain poorly understood. Long-standing efforts have been made to identify the IFN-induced proteins that are capable of mounting a response against HCV infection; research towards this aim has been hampered, however, by the lack of HCV cell culture and small animal models. The chimpanzee remains the only suitable animal model for studying HCV infection in vivo, which is, however, not widely applicable due to ethical and economical concerns. Efficient HCV replication in cell culture has only been available since 1999, when an HCV subgenomic replicon system in human hepatoma-derived Huh7 cells was established [30]. In 2005, an in vitro model of HCV replication that reproduces the full viral life cycle [31, 32], including production of infectious virus, was developed. These models represent useful tools for investigating the efficacy of IFNα and identifying potential IFN-induced effector proteins against HCV. It is assumed that Viperin and PKR are important players in the battle against HCV [33, 34]. Interestingly, HCV proteins were shown to interact with some of those IFN-induced antiviral effectors. For example, the two HCV proteins NS5A and E2 are known to interact with PKR [35, 36]. These interactions lead to an inhibition of PKR activity and therefore to an HCV-mediated IFNα resistance.

HCV has a striking capability to establish chronic infection, as 70–80% of infected individuals fail to eliminate the virus. Inhibition of the Jak-STAT pathway, which is crucial for most of the known antiviral effects of IFNs, might therefore be a central target of HCV providing an important advantage for the virus early in the course of infection. Indeed, our laboratory has previously reported that HCV proteins inhibit IFN-induced signalling in cell lines, transgenic mice and patients with CHC [37–39]. This inhibition was associated with an HCV-induced upregulation of the protein phosphatase 2A, finally resulting in STAT1 hypomethylation [39]. Other research groups have identified further mechanisms including inhibition of STAT1 activation through up-regulation of the negative regulator SOCS3 by HCV core protein [40] and proteasome-dependent degradation of STAT1 in Huh7 cells expressing HCV proteins [41]. Epidemiological studies show that the remaining 20–30% of patients overcome acute HCV infection, suggesting that innate and/or adaptive immune responses are indeed capable of controlling the outcome of HCV infection. Evidently, HCV cannot block IFN signalling
Analysis of interferon signalling in the liver as a basis to understand treatment non-responsiveness in patients with chronic hepatitis C

To learn more about possible mechanisms underlying differential response to IFNα-based therapy, we investigated IFN-induced signalling and ISG induction in paired liver biopsies collected from patients with CHC before (B-1) treatment and 4 hours after (B-2) the first pegIFNα injection. In addition to the liver samples, blood samples for isolation of peripheral blood mononuclear cells (PBMCs) were collected before (PBMC-1) and during (PBMC-2) therapy (see figure 3A for study outline) [46]. Virological response early in the course of treatment is an excellent predictor of later SVR. Early virological response (EVR; ≥2 log10 drop in 12 weeks) is associated with an SVR in 65% of cases [9]; a RVR leads to an even greater SVR rate of 80–90% [10, 47]. We therefore divided the 16 patients, from which paired liver biopsy samples were obtained, into rapid responders (RRs; n = 10) and non-RRs (n = 6) at week 4 of treatment. The aims of the study were: (i) to analyse IFN-induced effects in liver and PBMCs, (ii) to correlate IFN-signalling data with virological response to treatment, and (iii) to understand the molecular basis for the failure of IFN therapies in patients with CHC.

Besides infecting humans, HCV can infect chimpanzees. Interestingly, however, chimpanzees do not respond to IFN-based treatment [48] and are therefore not a suitable model for studying the molecular basis underlying treatment failure. Furthermore, chimpanzees chronically infected with HCV have numerous ISGs induced in the liver [49]. Injection of pegIFNα into these animals does not result in a further increase of ISG expression in liver, unlike in PBMCs which show a robust response to IFN [48]. These findings point to the importance of tissue-specific differences when studying the HCV infection in humans. Many studies have been performed using PBMCs from CHC patients, but it is unknown whether results obtained with PBMCs can be extrapolated to IFN-induced events occurring in the liver, the major site of HCV replication. Similarly to chimpanzees, in many CHC patients the endogenous IFN system is activated in liver tissue collected prior to treatment [46, 50–53]. Importantly, there is a large variation in the pre-treatment level of ISG expression among patients, and the degree of activation of the endogenous IFN system is linked to the patients’ response to IFN therapy. Patients with high expression of ISGs measured in pre-treatment liver biopsies represent poor responders, whereas those lacking pre-activation of ISGs show a good response to therapy [46, 51]. These findings are in line with the chimpanzee data, though they are counterintuitive, as one would expect patients exhibiting elevated IFN response to clear the virus more rapidly.

Prior to our work, no data involving repeated liver biopsies from CHC patients undergoing treatment were available. By analysing the phosphorylated form of the transcription factor STAT1 (p-STAT1) (p-STAT1) in the paired liver samples (B-1 and B-2) from 16 patients [46], we observed that nuclear translocation of p-STAT1 in the RR patient group occurs only after treatment with pegIFNα, whereas in the non-RRs p-STAT1 is already present in hepatocyte nuclei in the B-1 liver and no further activation is observed in response to the pegIFNα injection (figure 3B). In contrast to hepatocytes, other cells in the liver (e.g., Kupffer cells and lymphocytes) respond normally to the pegIFNα treatment in both RR and non-RR patients. These results indicate that some CHC patients do not benefit from the pegIFNα administration, as their hepatocytes linger in a pre-acti-
activated and likely “refractory” state. In the other group of patients (i.e., the future responders), infection with HCV seems not to be sensed by the liver in terms of activation of IFN signalling. In these patients, pegIFNα treatment succeeds to eliminate HCV possibly as a result of the antiviral defence system being rapidly mounted and confronting a virus that has not yet adapted to coping with an IFN response.

Microarray analysis of gene expression at the mRNA level revealed pronounced differences between the RR and non-RR patient groups in the number of genes responding to pegIFNα in the liver. We compared B-1 with B-2 liver samples of each individual patient, and found substantially more IFN-regulated mRNAs in the RR patient group. In PBMCs from the same patients, many more genes were regulated by pegIFNα than in liver, but, at the same time, differences between the patient response groups were far less pronounced. These observations argue for a strong local defect in the IFN-induced signalling pathways in the liver. Figure 3C depicts the typical ISG mRNA expression pattern, exemplified by Mx1 and Viperin genes, seen in the investigated patient samples; it underscores the differences between response groups and between analysed tissues.

We also analysed a large cohort of 112 pretreatment liver biopsies and found that patients infected with “difficult-to-treat” HCV GTs 1 and 4 more often have a pre-activated endogenous IFN system prior to treatment, when compared to patients with HCV GTs 2 and 3. This observation might provide an explanation for the worse treatment prognosis of patients infected with GT 1 and 4. Why the endogenous IFN system is activated in a considerable group of patients but not in all of them is not clear. This could be due to the differences between viral GTs and quasispecies in their capacity to prevent the endogenous IFN induc-

Figure 3
IFN signalling and treatment outcomes in chronic hepatitis C.
A. An initial liver biopsy (B-1) and an initial PBMC sample (PBMC-1) were obtained during diagnostic examination of patients with CHC. The second biopsy (B-2) and a second sample of PBMCs (PBMC-2) were collected 4 hours after the first dose of pegIFNα. After 4 weeks of pegIFNα and ribavirin combination therapy, patients were defined as rapid responders (RRs) or non-RRs depending on the drop in HCV viral load (VL). B. We performed immunohistochemical staining of the activated transcription factor STAT1 in liver biopsy samples. Shown are representative examples of B-1 and B-2 of RR and non-RR patients. No nuclear staining is evident in pre-treatment biopsies of RR patients (Patient 4). The light blue colour of the nuclei originates from the counterstaining with hematoxylin. Four hours after pegIFNα, most hepatocytes show strong nuclear staining (in brown). In non-RR patients (Patient 12), weak nuclear staining is already present in pre-treatment biopsies, and pegIFNα induces little change in hepatocytes. The visible increased nuclear staining is confined to Kupffer cells. C. Gene expression pattern of two classical ISG mRNAs (Mx1 and Viperin). Expression in B-1 samples of RR patients (lanes 5–14) resembles healthy control tissue (lanes 1–4), whereas ISG levels in B-1 samples of non-RRs (25–30) are elevated. PegIFNα treatment increases the ISG mRNA level in B-2 samples in the RR (15–24), but not the non-RR (31–36) patient group. PBMC-1 and -2 samples do not differ substantially between RRs (37–56) and non-RRs (57–68). (Reprinted with permission from Sarasin-Filipowicz et al., Proc Natl Acad Sci U S A 2008;105:7034–9. Copyright 1993–2008 by the National Academy of Sciences, Washington, DC).
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Are microRNAs important players in HCV infection?

MicroRNAs (miRNAs) should also be considered when discussing HCV infection. MiRNAs are short (21–23 nucleotide-long) regulatory RNAs expressed in metazoan animals [56]. They regulate gene expression by base pairing to target mRNAs and inhibiting protein synthesis [57]. MiRNAs are involved in the control of nearly all cellular processes and over 50% of all human mRNAs are predicted to be miRNA targets [58].

There is evidence that miRNAs play a role in viral infections and also in the innate immune response. Some viruses, such as CMV or human herpes virus-8, encode miRNAs in their own genomes, and these miRNAs may modify expression of the host's genes [59]. In contrast, some of the host-encoded miRNAs may have a profound effect on the life cycle of the infecting virus. MiR-122, a very abundant human miRNA, is expressed specifically in hepatocytes, and is the most spectacular example of the latter category. MiR-122 base pairs to genomic RNA of HCV and positively regulates replication of the virus in cell culture [60, 61]. This observation raised much interest in the role of miR-122 in HCV infection and as a potential therapeutic target. Recently, it was reported that the expression of miR-122 and several other miRNAs is regulated by IFN in Huh7 cells and primary mouse hepatocytes, and that these miRNAs might mediate at least some effects of IFN on HCV-RNA replication in vitro [62].

Therefore, we studied the status of miR-122 and other implicated miRNAs during the course of HCV infection and following IFNα therapy in CHC patients [63]. The availability of biopsy material collected for the studies discussed above [46] allowed us to study the proposed connection between miR-122 and HCV replication in a context of diseased tissue, and to test the effect of pegIFNα administration on the levels of miR-122 and other miRNAs in human liver.

Figure 4 summarises the expected results and our actual findings. If miR-122 were required for efficient HCV replication in vitro as it is in Huh7 cells [60], low hepatic miR-122 levels should lead to low HCV VL. Additionally, since low baseline VL is associated with a favourable response to therapy [8, 9], we were expecting to find low levels of miR-122 in good responders (fig. 4, left panel). Indeed, major differences were found in miR-122 levels between individual patients with CHC. However, rather unexpectedly, pre-treatment levels of miR-122 were markedly lower in patients who later showed no response to pegIFNα therapy (PNR patients). Moreover, we found no positive correlation between miR-122 abundance and intrahepatic or serum VL (fig. 4, right panel). It is possible that even the low miR-122 level found in PNRs is sufficient for robust HCV replication. Alternatively, the role of miR-122 in HCV replication may be less pronounced in vivo than in vitro.

It is currently unknown why PNRs have lower miR-122 levels than cEVR patients. We discussed earlier that PNR patients have a pre-activated IFN system in the liver already before treatment and that they show no significant changes in expression of IFN-regulated genes upon pegIFNα administration [46]. The decrease in miR-122 level in PNRs raised the possibility that miR-122 is a negatively regulated IFN target gene. Indeed, it has previously been reported that miR-122 is downregulated by treatment with IFNβ and that this downregulation contributes to the antiviral effect of IFN in Huh7 cells [62]. We compared the levels of miR-122 in paired liver biopsies collected before (B-1) and 4 hours after (B-2) administration of pegIFNα but found no decrease in the level of miR-122 in any of the 11 patients investigated. We can, however, not exclude that miR-122 is a late IFN-regulated gene in the human liver. The other miRNAs that we analysed were either not IFN regulated and not expressed at biologically relevant levels in human liver. Therefore, it is
very unlikely that miRNAs mediate antiviral IFN responses against HCV.

Is the role of miR-122 in HCV replication less important in vivo than it is in vitro? This question will be answered by ongoing trials in HCV-infected chimpanzees in which antisense oligonucleotides blocking miR-122 activity, that is miR-122-“antagomirs”, are being evaluated as anti-HCV agents. If strategies aimed at reducing hepatic miR-122 levels prove to be successful, these “antagomirs” may be particularly promising in PNRs because of their low baseline miR-122 levels.

The future of anti-HCV therapy

Current standard treatment of CHC has known limitations given the high number of patients without response and the frequent occurrence of side effects. New prospects are created by introduction of the specifically targeted antiviral therapy for HCV (STAT-C), such as small molecule inhibitors of viral proteins. Currently, two HCV NS3-4A protease inhibitors are being evaluated in phase III clinical trials: Telaprevir (VX-950) and boceprevir (SCH 503034) [64, 65]. Inhibition of the HCV NS3-4A protease might not only decrease viral replication but also enhance antiviral innate immune pathways (as cleavage of MAVS is prevented, see above) [44]. Various nucleoside and non-nucleoside HCV RNA polymerase inhibitors are also being investigated. It is expected that small molecule inhibitors will soon be part of standard anti-HCV therapies. A major challenge will be the inevitable occurrence of viral resistance and frequent side effects, such as rashes and anemia [64]. Administration of a single protease or polymerase inhibitor leads to the development of resistant viral strains, as rapidly as 1–2 weeks after treatment initiation [66]. To overcome development of resistance, administration of “cocktails” of various inhibitors can be considered; the limiting factor will, however, be the high toxicity of such treatments. Also, when these novel molecules are combined with current standard treatment, resistance can be avoided. Early results from phase 2b trials show that SVR rates in GT1 patients increase from 40–50% to 67–75% when triple combinations are used [64, 65, 67]. The novel therapeutics may have potential to improve and shorten the duration of therapy; they will, however, not replace the use of pegIFNα and ribavirin. It is evident that non-responders to pegIFNα-based treatment will have an increased risk for the development of viral resistance because of persistently high HCV levels. Should it be possible to reverse pre-activation of the endogenous IFN system in the liver (by using immune modulators or IFN antibodies) non-responders might be turned into responders and many more patients could be cured. Research towards that aim is urgently needed.

Another strategy is to modify IFNs in order to increase efficacy or decrease toxicity. This approach has proven to be successful in the past with the introduction of pegIFNα. However, the newly developed albumin-modified IFNα is unlikely to improve treatment outcomes, although it can be less frequently administered thus elevating life quality of the patients [68]. IFN signalling can be enhanced by additional use of the methyl-group donor S-adenosyl-methionine (SAMe) [69]. HCV protein-induced hypomethylation of STAT1 can be reversed by SAMe in cell culture and ongoing clinical trials show promising results when retreating previous non-responders (ClinicalTrials.gov, Identifiers: NCT00310336 and NCT00475176).

Due to the limited response to treatment and the frequent side effects, prediction of response prior to treatment initiation would be very useful. Though factors like GT, baseline VL, and histological staging are well-characterised predictors of response, foreseeing treatment responses in individual patients remains difficult. So far, VL at week 4 of treatment is the most valuable criterion. It remains to be seen, whether a predictive test based on measurement of selected mRNAs in liver, as markers of pre-activation of the endogenous IFN system, will be of diagnostic use [46].

The results of studies with novel therapeutic compounds so far indicate that pegIFNα and ribavirin will remain the backbone of antiviral therapy of CHC even in the era of STAT-C. It can be anticipated that combination of STAT-C inhibitors with non-overlapping resistance profiles will further improve response to antiviral treatment.
The development and validation of tests for the prediction of response to the different combination therapies that will be used in the near future would allow physicians to tailor the treatment to the needs of individual patients.

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