Cellular stress response during hepatitis C virus infection
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Discussion, Summary and Perspectives
Introduction

Since the isolation of the hepatitis C virus (HCV) genome in 1989 (1), research on HCV biology and pathogenesis has progressed rapidly and today we are witnessing the ambition of the World Health Organization (WHO) to eliminate viral hepatitis before the year 2030 (2). However, despite these advances there are still gaps in our knowledge on this virus that may challenge this goal. HCV pathogenesis is complex and not completely clear yet. In particular, the host-virus interaction requires additional research, since it determines to a large extent the outcome of HCV infection and the clinical management of infected individuals.

The research described in this thesis focused on aspects of host-virus interaction: the impact of viral protein expression on adaptive mechanisms in the host cell (hepatocyte). More specifically, we investigated the molecular mechanisms involved in the adaptive response to cellular stress in hepatocytes expressing HCV viral proteins while subjected to an additional stressor: oxidative stress. This is an important aspect in HCV research as HCV-infected hepatocytes have to cope simultaneously with 1) HCV replication and expression of viral proteins and 2) inflammatory signals and oxidative stress. We developed a cell culture model to mimic these stresses. Additionally, we used a co-culture system of Huh7 cells expressing HCV proteins and hepatic stellate cells (HSC) to study the interaction between virus-infected cells (hepatocytes) and fibrogenic cells (HSC). The study of this interaction is highly relevant, since activated HSC are the principal fibrogenic cell type. In Figures 1 a graphical abstract of the models used in this thesis is presented together with the major findings. Initially, we used Huh7 cells with transient expression of HCV Core or NS3/4A proteins (viral proteins with pro-oxidative activity) to study the adaptations to external oxidative stress exposure (menadione treatment) (Figure 1A). We observed that Huh7 cells expressing HCV proteins resist the pro-apoptotic effect of external oxidative stress (Chapter 3). Then, the molecular mechanism of resistance to external oxidative stress was unraveled and described in Chapter 4 using Huh7 cells with stable expression of HCV proteins (Figure 1B). Finally, the co-culture of hepatocytes and HSCs was presented to study activation of HSC under oxidative stress conditions (Figure 1C).
Figure 1. Summary of the main results obtained in this thesis. A. Huh7 cells with transient HCV Core or NS3/4A protein expression can overcome oxidative stress-effects, through reduction in ROS production, apoptosis and ER stress markers, therefore, survival of Huh7 cells increased. B. The molecular mechanism behind oxidative stress resistance in Huh7 cells expressing HCV viral proteins were elucidated. After oxidative stress induction and HCV protein expression, eIF2α was phosphorylated and further ATF4 and CHOP expression were observed, thus, eIF2α/ATF4 pathway was activated. Autophagy markers were suggested to play an important role during HCV Core or NS5A degradation and concomitant cell survival. C. Cellular stress response was determined also in HSC after co-culture with Huh7 cells expressing HCV Core and NS3/4A.

From our results, we conclude that the adaptive response to cellular stress (specifically to oxidative stress) could play an important role in the outcome of HCV infection (Figure 2). In the acute phase of HCV infection, the adaptive response to cellular stress may promote virus resolution as consequence of degradation of pro-oxidant HCV proteins as Core and NS5A, which will limit virus production. Reduced virus production may benefit the hepatocytes, since it prevents the death of host cells (and hence a reduction in virus ‘factories’). In chronic infection, we suggest that the adaptive response to cellular stress in HCV-infected hepatocytes can be modulated by HCV viral proteins which may benefit viral persistence (Figure 2). From the point of view of the hepatitis C virus: ‘What doesn't kill me, makes me stronger’.
Effects of HCV protein expression on mitochondria, ER stress and cell death

In Chapters 3 and 4, we report that HCV protein expression increases the production of mitochondrial reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and apoptotic cell death. These phenomena are most likely interrelated: HCV replication involves the rearrangement of intracellular membrane systems, including the ER, mitochondria and other organelles, to establish the membrane-associated replication complex (Figure 3 in Chapter 1) (3). The specific modifications at the ER are mediated by various non-structural HCV proteins, such as NS4B and NS5A that are localized inside the ER lumen or integrated in the ER membrane (4). HCV also uses components of the intracellular lipid transport system for the production of new infectious viral particles (5). Thus, HCV infection disrupts normal ER function and induces ER stress. In turn, ER stress may result in increased oxidative stress (6) and, together with ROS generated during the immune response against HCV, impose significant oxidative stress on the hepatocytes, resulting in apoptotic cell death (7,8).

The ER is responsible for protein synthesis and protein quality control (6). Elimination of misfolded proteins occurs through activation of the ER-associated protein degradation pathway and the ubiquitin-proteasome pathway (9). Autophagy also plays an important role in sequestering misfolded proteins inside ER-derived membrane vesicles (10) and subsequent degradation of these misfolded proteins (11). Thus, ER stress and autophagy are intertwined cellular mechanisms. Overload of these systems, e.g. by misfolded or aberrant proteins, induces ER stress and initiates the Unfolded Protein Response (UPR). In our studies, we demonstrate the involvement of several components of the UPR in response to HCV protein expression, notably double-stranded RNA-activated protein kinase (PKR)–like ER kinase (PERK) signaling, via its downstream effectors alpha-subunit of the eukaryotic translation initiation factor 2 (eIF2a), Activating Transcription Factor 4 (ATF4) and C/EBP homologous protein (CHOP) (Chapter 3 and 4).
Increased resistance to external oxidative stress in hepatocytes expressing HCV core and NS3/4A

Several HCV proteins are known to induce or enhance ROS production directly. Core is reported to have the highest pro-oxidant capacity, but E1, E2, NS3, NS4B and NS5A have all been shown to promote ROS production in hepatocytes (8-14). We analyzed the role of three important pro-oxidant HCV proteins, Core, NS3/4A and NS5A, in our in vitro model.

In Chapter 3, we show that Huh7 cells and hepatocytes transiently expressing the pro-oxidant viral proteins Core or NS3/4A are more resistant to apoptosis induced by external oxidative stress (menadione treatment): these cells demonstrate reduced ROS production, ER stress (glucose-regulated protein 78kDa [GRP78], spliced X-box
binding protein 1 [sXBP1]) and apoptotic cell death (caspase-3 activity). Interestingly and contrary to earlier reports, we do not see a direct effect on general ROS production in cells expressing HCV Core, NS3/4A or NS5A. However, mitochondrial superoxide anion production and expression of the oxidative stress-marker Heme Oxygenase-1 (HO-1) encoded by HMOX-1 gene was significantly increased in Huh7 cells and primary hepatocytes transfected with HCV Core protein (Figure 1 and 3B in Chapter 3). Increased resistance of Core-expressing cells to cell death induced by oxidative stress has been reported before and shown to be dependent on inhibition of p53-mediated apoptosis (12). These results are different from ours and may be related to the use of different inducers of oxidative stress: hydrogen peroxide versus superoxide anions (menadione), leading to different modes of cell death, e.g. necrosis and apoptosis, respectively (13). Several studies have been performed to explore the direct pro-oxidative role of HCV proteins in HCV infection, while other studies focused on the anti-oxidative response after HCV protein expression. The results suggest a dual effect: HCV proteins induce oxidative stress, resulting in activation of the Nuclear factor [erythroid-derived 2]-like/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) pathway and increased cell survival (14–17).

In Chapter 3, we demonstrate significantly reduced levels of p62/sequestosome 1 (SQSTM1) protein (hereafter referred to as p62) and increased levels of the phosphatidylethanolamine-conjugated form of microtubule-associated protein 1A/1B-light chain 3 (LC3-II) in Huh7 cells expressing HCV Core or NS3/4A. The degradation of HCV Core protein correlates with the decrease in p62 and suggests a correlation between activation of autophagy, degradation of Core protein and the resistance to oxidative stress. The effects on p62 and LC3-II are potentiated by exposure to external oxidative stress (Figure 5B in Chapter 3). Additional studies are required to demonstrate a causal relationship between p62 and Core degradation, autophagy and resistance to oxidative stress in the model of transient expression of HCV viral proteins in Huh7 cells (Chapter 3).

Another intriguing finding reported in Chapter 3 is that the adaptive response to cellular oxidative stress is abolished by the anti-oxidant N-acetyl-L-cysteine (NAC). This indicates that some level of ROS production is necessary to activate the adaptive response. This observation is very similar to the phenomenon of ‘pre-conditioning’ in which toxic effects of a specific stressor can be prevented by a
priori exposure to low (non-toxic) levels of the same stressor. In fact, HO-1 has been shown to be instrumental in the protective effect of pre-conditioning in the context of ischemia-reperfusion injury (18,19). A similar phenomenon may be operational in our external oxidative stress induction model, in which HO-1 expression is also increased. Interestingly, HO-1 is a Nrf2 target gene. The Nrf2-Keap1 pathway, also defined as Nrf2/Antioxidant Response Elements (ARE) pathway, is the major regulator of a cytoprotective response to oxidative stress. Burdette et al. investigated the mechanism of Nrf2 activation in human hepatoma cells transfected with an HCV-replicon. Activation and nuclear translocation of Nrf2 was observed and this was inhibited by the antioxidant pyrrolidine dithiocarbamate (PDTC) and the Ca\(^{2+}\)-chelator 1,2-bis (aminophenoxy)ethane N,N,N,N-tetraacetic acid tetra(acetoxy methyl)ester (BAPTA-AM), suggesting a role for both ROS and Ca\(^{2+}\) signaling in Nrf2-activation (16). Phosphorylation of glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) is correlated with Nrf2 activation in liver biopsy specimens from HCV-infected patients and inversely correlated with the degree of liver injury. Since GSK3\(\beta\) is an indispensable regulator of the oxidative stress response, therapeutic targeting of GSK3\(\beta\) has been proposed to enhance the antioxidant Nrf2-dependent response during chronic HCV infection (20). Although we did not specifically investigate the Nrf2-Keap1/GSK3\(\beta\) pathway, it may be a relevant topic for future studies.

In addition to HO-1, other antioxidant enzymes that detoxify ROS were evaluated, such as superoxide dismutase (SOD1 and SOD2), catalase (CAT) and glutathione peroxidase 1 (GPx1). We did not observe any changes in the expression of these genes, indicating that the observed resistance to oxidative stress is not mediated by transcriptional regulation of these enzymes (Figure 2 in Chapter 3; Supplementary Figure 2 in Chapter 4). This is in line with previous studies reporting a lack of induction of antioxidant proteins such as CAT, Nqo1, and GPx1 in a HCV acute infection model (21,22).

**Adaptive mechanisms in hepatocytes to external oxidative stress induction**

In Chapter 4, we explore the molecular mechanism(s) contributing to the resistance to oxidative stress in Huh7 cells stably expressing these HCV proteins, mimicking chronic infection. We demonstrate activation of the eIF2a and subsequent expression
of transcription factor ATF4 in response to oxidative stress and the selective
degradation of Core and NS5A HCV proteins, resulting in a reduction of cellular
oxidative stress (Figure 3 in Chapter 4). ATF4 has an important role in regulating both
normal metabolic and redox processes, as well as acting as a master transcription
factor during the Integrated Stress Response as described in Chapter 2 and in (23).
ATF4 regulates the transcription of target genes like ATF5 and DDIT3 via
binding to the C/EBP-ATF response element (CARE) (24).

The expression of DDIT3, the gene encoding CHOP, is induced in Huh7 cells expressing
Core or NS5A. Similarly, in liver samples of patients infected with HCV, the increased
expression of ATF4 and DDIT3 is also observed. The increased expression of these
markers is restricted to the cirrhotic stage of HCV infection and is not observed in
samples from HCV-associated hepatocellular carcinoma and non-HCV-related liver
diseases (Figure 4 in Chapter 4). The specificity of ATF4/DDIT3 induction for HCV-
related liver disease is striking and supports our in vitro data. In line with our results,
increased expression of ATF4 and CHOP and modulation of the steady state of
autophagy markers has been reported in Huh7 cells expressing HCV Core protein
(25). Furthermore, HCV-infected Huh7.5.1 cells and HCV-transgenic mice (expressing
the entire HCV genotype 1b genome under alpha-1 antitrypsin promoter) display
increased phosphorylation of eIF2a and induction of downstream genes like ATF4 and
DDIT3. In the HCV-transgenic mice, suppression of HCV replication by interferon-a2a
treatment normalized ATF4 and DDIT3 expression (26).

Several findings suggest a role of the eIF2a/ATF4 pathway in regulating autophagy in
response to stress (27,28). Important autophagy-related target genes of the eIF2a/
ATF4 pathway are the cargo receptors that are involved in the specific degradation of
ubiquitinated substrates, such as p62 and Neighbor of BRCA1 gene 1 protein (Nbr1)
(27). p62 is a conserved, stress-inducible multifunctional protein that acts as a receptor
for ubiquitinated cargos in autophagy (29). p62 has several domains that mediate its
interactions with various binding partners involved in selective autophagy, nuclear
factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation, apoptosis,
mammalian target of rapamycin complex 1 (mTORC1) activation, oligomerization,
adipogenesis and the Nrf2/Keap1 pathway, making it an important signaling hub (30–
33).
p62 is mostly localized in the cytoplasm, however, it can also be found in the nucleus, autophagosomes and in lysosomes. In response to cellular stress, p62 translocates to autophagy substrates, such as protein aggregates and damaged proteins and organelles, like mitochondria. Cytoplasmic p62 is removed mainly by autophagy and its presence in the cytoplasm is generally considered to inversely correlate with autophagic activity (34). Presence of p62 is associated with a basal level of autophagy, whereas accumulation of p62 is used as an indicator of impaired autophagy (34).

Since p62 acts as an autophagy receptor for ubiquitinated cargos and based on the results presented in Chapter 3, we hypothesize in Chapter 4 that p62 may play an important role in HCV Core and NS5A degradation and the resistance to oxidative stress. Levels of p62 are strongly downregulated after induction of oxidative stress in Huh7 cells stably expressing HCV Core, NS3/4A and NS5A proteins. Similar to the results observed in Chapter 3, degradation of HCV Core correlates with p62 degradation. In addition to elimination of Core, we also observed degradation of NS5A in stably transfected cells in Chapter 4. This phenomenon is specific for HCV Core and NS5A proteins, since the level of NS3/4A is not affected by exposure to menadione (Figure 5 in Chapter 4). A possible explanation is that HCV Core and NS5A are the major pro-oxidant factors compared to the other viral proteins and their elimination may therefore rescue the cells from oxidative stress-induced apoptosis (14). The involvement of p62 in HCV Core and NS5A degradation is confirmed using p62 silencing (Figure 8C and 8D in Chapter 4). Thus, HCV Core and NS5A can increase oxidative stress and the p62-dependent degradation of these proteins via selective autophagy may represent a new mechanism to reduce oxidative stress and prevent cell death, explaining the resistance to oxidative stress observed in our model of injury.

Autophagy is an important characteristic during HCV infection and it is now well established that HCV induces autophagy to support its own replication (35). Different studies have addressed the molecular mechanism(s) underlying the modulation of autophagy by HCV (35–38). Our studies suggest that selective degradation via lysosome of harmful viral proteins thereby preventing hepatocyte cell death.
Interaction between HCV-infected hepatocytes and hepatic stellate cells

In Chapter 5, we study cell-cell interaction between two important cell types involved in HCV-induced fibrogenesis, e.g. hepatocytes and HSC, using a transwell co-culture system of Huh7 cells expressing the HCV proteins Core or NS3/4A and HSCs. We confirm that Huh7 cells expressing HCV proteins activate HSCs as indicated by the increased expression of fibrogenic markers like Actin Alpha 2, Smooth Muscle gene (\textit{a-SMA}), Collagen Type I Alpha 1 Chain (\textit{COL1A1}) and transforming growth factor beta 1 (\textit{TGF-b1}) (39,40). The transwell co-culture system is more representative than the use of conditioned media to study the interaction between the different cell types involved in HCV-induced fibrogenesis, because of the close proximity of the cell types and the sustained release of any factors secreted by these cells, e.g. cytokines or profibrogenic factors. Other studies using conditioned media of Huh7 cells expressing HCV proteins have also shown the fibrogenic activation of HSC, but these studies used HCV-replicon Huh7 cells expressing HCV non-structural proteins (39), while we used transiently transfected Huh7 cells. These methodological differences may explain why we were not able to detect activation of HSC using conditioned media. Interestingly, induction of external oxidative stress to our co-culture model did not induce markers of oxidative stress or ER stress in HSC. This might be due to the fact that the oxidative stress is limited to hepatocytes (Huh7 cells), because the viral proteins are only expressed in Huh7 cells and menadione metabolism (generating ROS) is also mainly active in hepatocytes. These results demonstrate that the Huh7 cells, subjected to external oxidative stress, do not release any factors that induce oxidative stress or ER stress in HSC.

Concluding remarks and perspectives

In this thesis, we investigated the adaptive response of hepatocytes expressing HCV proteins to an additional stressor, oxidative stress. We focused on several aspects of the Integrated Stress Response in hepatocytes expressing the HCV proteins. Our main conclusion is that HCV Core and NS5A precondition hepatocytes to resist oxidative stress. The adaptation involves a p62-dependent mechanism of selective autophagy and reduction of the pro-oxidant viral proteins Core and NS5A. The consequences
of this adaptive response have to be carefully interpreted in the context of acute or chronic infection. During acute infection, the adaptive response to cellular stress, e.g. oxidative stress, could induce the degradation of harmful pro-oxidative proteins as Core and NS5A thereby limiting the viral infection. On the other hand, during chronic HCV infection the infected hepatocytes survive and infection persists, because of the modulation of the adaptive response to cellular stress by the expression of HCV proteins. Thus, this adaptive response is a double-edged sword, limiting liver injury (by protecting hepatocytes) and maintaining viral infection. Our results identify novel potential targets for intervention in viral infection (autophagy, ER stress), but also highlight the dilemma’s when intervening in processes that at the same time promote cell survival and viral replication.

We also observed an intriguing role for anti-oxidants in our model: the adaptive response is abolished by anti-oxidants. This could be related to the fact that the adaptive response is an example of ‘pre-conditioning’ in which low level exposure to a particular stress confers protection against a subsequent high-level exposure to the same stress. In this model, anti-oxidants would abolish the low-level exposure to ROS, preventing ‘pre-conditioning’. The effect of anti-oxidants also has clinical implications: the use of anti-oxidants to prevent oxidative stress in various liver diseases (HCV, non-alcoholic fatty liver disease) may not always be recommended and, in fact, be detrimental.

It is important to confirm and validate our findings in clinically relevant situations, i.e. in liver tissue of patients infected with HCV as well as in a full replication model of HCV in Huh7 cells. In this thesis, we demonstrate the increased expression of ER stress response genes (ATF4, DDIT3) in liver tissue of HCV-infected patients. Moreover, this increased expression appears to be specific for HCV infection. More extensive and detailed studies are necessary to confirm whether the adaptive mechanisms observed after external oxidative stress induction are also operative in clinically relevant HCV infection. Another interesting area of follow-up research may be the effect of ER stress relieving agents in our model: compounds like valproate, oleic acid and tauroursodeoxycholic acid (TUDCA) have been reported to attenuate ER stress. Testing these compounds in our model and, eventually, in a clinical setting would be of major interest.
References


