Expression pattern of molecular chaperones after liver transplantation in hepatitis C positive recipients. Relation to serum HCV-RNA titers

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Abstract: Hepatitis C (HCV) is one of the main causes of liver transplantation (OLT). Previously we have reported that high serum C RNA level correlates with the severity of histopathological signs and poor clinical outcome. The core antigen of virus C is known to interfere with chaperones in the hepatocytes, results in an endoplasmic reticulum (ER) stress. In this study HCV positive liver transplanted patients were evaluated, whether there are correlations among chaperone expression, recurrence and viral titer. Patients were enrolled after surviving the first month following OLT. Sera were collected regularly, and biopsies were taken on demand following OLT. The diagnosis of recurrent HCV was proven by Knodell-Ishak scoring. In this case ribavirin + interferon were initiated, and maintained for one year. All chaperones were upregulated in the transplanted liver graft showing recurrent hepatitis C disease. ATF6, GP96, GRP78, CNX and CLR chaperones were upregulated significantly compared to their levels in normal livers. Except for one chaperone, the level of upregulation did not correlate with the serum's HCV-RNA titre: the only difference between Group1 and 2 (RNA titre above and below 8.78 10^6 respectively) was that the level of ATF6 was 1.6 times higher in Group1 compared to Group2. The expression of all chaperones was reduced, and some even became downregulated after the interferon treatment. In accordance with the literature our results suggest that hepatitis C might induce apoptosis through ER-stress. Those cells exposed to a high C viral load, had a lower chance to be eliminated.

Keywords: liver transplantation, hepatitis C virus, chaperone, apoptosis, viral titer, RNA, recurrence, XBP, ATF6

Introduction

Hepatitis C is one of the main causes of liver transplantation. Recurrence of the viral disease is unavoidable, however, there are differences in the timing and clinical course. The prognostic value of the postoperative HCV-RNA titre in recurrent HCV infection following OLT is controversial [1, 2, 3]. We have reported previously the correlation of the high C-RNA serum level following OLT, the severity of histopathological signs and the poor clinical outcome [4]. This is consistent with other reports [5]. The role of apoptosis in the recurrence of HCV following OLT is known [6]. Hepatitis C virus is a positive-strand RNA virus which belongs to the *Flaviviridae* family [7]. The core antigen of the C virus is known to interfere with the chaperones in the hepatocytes, which results in an endoplasmic reticulum stress (ERS). Molecular chaperons are mostly residents of the endoplasmic reticulum (ER) membrane. They are responsible for proper folding of the de novo synthetised proteins. Un- or misfolded proteins stay in the lumen of ER causing ERS that results in the unfolded protein response (UPR). On one hand the UPR cascade activates apoptosis via the NF-κB system, but it also has an antiapoptotic effect. The focus of our interest was to determine whether there are differences in the expression of resident membrane chaperones between patients characterized by a high serum viral titre and low replication after OLT. We also evaluated whether the effect of interferon treatment can be detected in the chaperon expression. In order to determine this we have enrolled the same cohort of patients that we had evaluated in our previous study [4].

Patients, material and methods

Patients, biopsies, definition of the study group

All patients were enrolled after surviving the first month following OLT. Sera were collected 1, 2, 3, 6 and 12 months after OLT for HCV-RNA PCR (RT-PCR Roche version 2.0; expressed in million copy/ml). Genotypes were also detected [8]. Biopsies were taken after liver transplantation within 6 months on demand and/or at every year per protocol. The diagnosis of recurrent HCV was histologically proven by Knodell-Ishak scoring [9, 10]. In case of proven HCV recurrence ribavirin + interferon were initiated as soon as possible, and maintained for one year. By the end of the 52nd week of IFN treatment a control biopsy was taken, and was also evaluated as mentioned above. If the recurrence was detected within 1 year postOLT we defined it as early viral recurrence, while late recurrence was considered when it occurred over the first 12 months. We determined groups by the serum HCV-RNA titres. A cut off point was set by a receiver operating curve (ROC) analysis that had been performed (SPSS Programme 13.0) between early and late recurrence using the highest measured HCV-RNA titre within the first 6 months. This proved to be 8.78×10^6 copy/ml (area: 0.84; specificity: 77%; sensitivity: 95%; $p = 0.009$), meaning that patients with an early recurrence had higher HCV-RNA titer (Group1) while patients with a late recurrence had a lower HCV-RNA titer (Group2). As we have reported earlier [4] there were no statistical differences between the two Groups with regards to donor age, gender and volume of transfusion given to the donor, as well as the type of organ perfusion fluid (University Wisconsin-UW or hystidin-triptophan-ketoglutarate-HTK). Furthermore no differences were found between the groups in recipient age, gender, Child-Pugh score, transfusion during operation; postoperative bleeding and technical complications (vascular, biliary) and in the need for renal replacement therapy. In *Group1* more patient had acute rejection episode (53% vs. 22%; $p = 0.04$) with a consecutive anti-rejection bolus treatment (53% vs.17%; $p = 0.02$). Infectious complications (69% vs. 31%; $p = 0.007$) were statistically more common in Group1 than in Group2. Sepsis was also more common (75% vs. 25%; $p = 0.12$) without a statistical relevance.

Thirty-six (36) formalin-fixed, paraffin-embedded biopsy specimens from liver transplanted patients (28 cases with low HCV titre and 8 cases with high HCV titre) were analyzed by molecular biological methods. Five (5) pieces of normal liver tissue obtained from healthy individuals who had died in an accidents were used for control. The elapsed time between death and tissue fixation was 6 to 12 hours. All samples were taken with informed consents of the patients in accordance with the Helsinki declaration and with the permission of the local ethical committee.

Molecular biology methods

Real-Time polymerase chain reaction (RT-PCR) analysis of XBP1 (x-box protein) total, XBP1 spliced, ATF6 (activating transcription factor 6), HSP27 (heat-shock protein 27), GP96 (glycoprotein of 96 kDa), GRP78 (glucose-regulated protein 78), CNX (calnexine), CLR (calreticuline) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression.

RNA Isolation

Total ribonucleic acid (RNA) from formalin-fixed, paraffin-embedded biopsy specimens was isolated with High Pure RNA Paraffin Kit (Roche, Indianapolis, Indiana, USA), after eight pieces of 5-µm-thick sections were cut from each tissue block. Proteinase K (Applied Biosystems) digestion time was 16 hours for each sample. All purifications were performed in accordance with the manufacturer's protocol. After total RNA isolation, samples were kept at –80°C until further use. Total RNA integrity was verified.

Reverse Transcription of RNA

Total RNA (500 ng) was reverse transcribed for 50 minutes at 42°C in 30 µl with MulV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) in the presence of RNase inhibitor (Applied Biosystems) using Random Hexamers (Applied Biosystems).

Real-Time Quantitative RT-PCR

Real-time PCR reaction was performed with 2 µl cDNA templates in a total volume of 25 µl, using ABI Prism 7000 sequence detection system (Applied Biosystems). Each PCR was conducted in 25 µl volume of SYBR Green Supermix (Bio-Rad, Hercules, CA, USA, 1708851). Primary sequences (primers) were as shown in Table I.

Each PCR reaction was done in duplicate in 96-well plates, for 2 minutes at 95°C for initial denaturing, then 40 cycles at 95°C for 20 seconds, at 60°C for 30 seconds and 72°C for 1 minute. Data analysis and statistical evaluation were performed by REST (relative expression software tool, www.gene-quantification.info), using GAPDH as a reference gene. Relative expression was calculated by the aforementioned software, according to the following (simplified) equation:

$$R = \frac{E\,(\text{target gene})^{\Delta CP\,(\text{mean control} - \text{sample control})}}{E\,(\text{reference gene})^{\Delta CP\,(\text{mean control} - \text{sample control})}}$$

R = relative expression; E = effectiveness of PCR reaction (the multiplication factor of PCR increase/cycle, which ideally would be 2, representing the complete duplication of genetic material in each cycle); DCP = crossing point, the number of cycles, when the amount of RNA exceeds the threshold value

Table I Chaperone primer sequences used for PCR reactions

Chaperone	Primer	No. of basis pairs
XBP1total F	5'CATGGCCTTGTAGTTGAGAACCA-3' (378-400)	95
XBP1total R	5'ACTTCATTCCCCTTGGCTTCC-3' (452-472)	
	GI: 18148379	
XBP1spliced F	5'TGAGTCCGCAGCAGGTGC-3' (494-511)	81
XBP1spliced R	5'TCTGAAGAGTCAATACCGCCAGA -3' (552-574)	
	GI: 18148381	
ATF6 F	5'TGCTTCACCCAGAAGTTATCAAGAC -3' (1705-1729)	92
ATF6 R	5'GCAGGTGATCCCTTCGAAATG -3' (1796-1776)	
	GI: 56786156	
HSP27 F	5'CGTGTCCCTGGATGTCAACC -3' (395-414)	87
HSP27 R	5'TCGTGCTTGCCGGTGATC -3' (464-481)	
	GI: 4996892	
GRP78 F	5'ACCATCCCGTGGCATAAACC -3' (1353-1372)	108
GRP78 R	5'ACATCAAGCAGTACCAGGTCACCT -3' (1437-1460)	
	GI: 18044380	
GP96 F	5'TAAGCTCTATGTGCGCCGTGT -3' (1332-1352)	122
GP96 R	5'TCTCGCGGGAAACATTCAAG -31 (1434-1453)	
	GI: 4507676	
Calnexin F	5'CTCCCAAGGTTACTTACAAAGCTCC-3' (275-299)	89
Calnexin R	5'AATCCACCCTGACAGAGTTCCTC-3' (341-363)	
	GI: 31542290	
Calreticulin F	5'TGTTTGGTCCCGACATCTGTG-3' (460-480)	102
Calreticulin R	5'CCTTGCAACGGATGTCCTTG-3' (542-561)	
	GI: 5921996	
GAPDH F	5'CATTGACCTCAACTACATGG-3' (186-205)	121
GAPDH R	5'GAAGATGGTGATGGGATTTC-3' (287-306)	
	GI: 7669491	

F: forward, R: reverse

Results

Chaperone expressions are shown in Table II. All chaperones were up regulated in the transplanted liver graft tissues showing recurrent hepatitis C disease. ATF6, GP96, GRP78, CNX and CLR chaperones were upregulated significantly compared to their levels in normal livers. Except for one chaperone, the level of upregulation did not correlate with the serum's HCV-RNA titre: the only difference between Group1 and 2 (RNA titre above and below 8.78 10^6 respectively) was that the level of ATF6 was 1.6 times higher in Group1 compared to Group2. (5.8 vs. 3.7; $p = 0.05$) This difference disappeared after interferon treatment. A homogeneous difference was found comparing biopsies taken before and after a year of interferon treatment: the relative expression of all chaperones was reduced. Moreover total and spliced XBP, as well as HSP27 became downregulated during interferon treatment. (See Table II and Figure 1). On the other hand no difference was found between patients with previously high and low RNA replication.

Discussion

Hepatitis C virus (HCV) is a positive-strand RNA virus which belongs to the *Flaviviridae* family. Its genome contains a long open reading frame of 9,030 to 9,099 nucleotides that is translated into a single polyprotein of 3,010 to 3,033 amino acids. The viral genome is framed by a non-readable region on both 5' and 3' ends [11]. There is an untranslated region (5-UTR) on the 5' terminal of the genome which is a characteristic of hepatitis C virus. The HCV 5'-NCR also functions as an *internal ribosome entry site* (IRES) permitting cap-independent translation [12]. Cleavages of this polyprotein are co- and posttranslational and generate at least ten polypeptides including 2 glycoproteins, E1 and E2. HCV glycoproteins E1 and E2 interact to form complexes. Characteri-

Table II Relative expression of chaperones (compared to normal liver) in the two groups, before and after interferon treatment following liver transplantation (+ means upregulation and – means downregulation)

	HCV-RNA titer above 8.78 × 106 copy/ml compared to normal healthy liver sample		HCV-RNA titer under 8.78 × 106 copy/ml compared to normal healthy liver sample	
	Before IFN	After IFN	Before IFN	After IFN
	Relative expression (p value)		Relative expression (p value)	
XBP1 total	+1.60 (ns)	–1.07 (ns)	+1.86 (ns)	+1.86 (ns)
XBP1 spliced	+1.53 (ns)	–1.07 (ns)	+2.48 (ns)	+1.26 (ns)
ATF6	**+5.82 (0.001)**	**+2.85 (0.03)**	**+3.69 (0.001)**	**+3.42 (0.03)**
HSP27	+1.19 (ns)	–1.05 (ns)	+1.25 (ns)	–1.11 (ns)
GP96	**+5.35 (0.001)**	**+3.04 (0.04)**	**+5.06 (0.001)**	**+3.46 (0.02)**
GRP78	**+2.28 (0.01)**	**+1.35 (ns)**	+3.34 (0.002)	**+2.30 (ns)**
Calnexin	**+2.89 (0.01)**	**+1.71 (ns)**	+3.29 (0.001)	**+1.68 (ns)**
Calreticulin	**+3.11 (0.009)**	**+1.71 (ns)**	+2.43 (0.003)	**+1.99 (ns)**

zation of HCV glycoprotein complex formation indicated that a majority of these proteins are misfolded aggregates and their tendency toward aggregation could be an intrinsic property of HCV glycoproteins [7]. The UTR region is the most stable region of the genome, therefore this is the common target for genotype and serum RNA evaluations. The native virus polyprotein is dissected by its own proteinases. This is how the structural and non-structural (enzyme) proteins born. The virus' proteinss are the following: core (capsid) protein, E1 (envelop), E2, NS2, NS3, NS4a, NS4b, NS5a, and NS5b. The core protein suppresses the apoptotic process in vitro [13]. There is an association between the mutations of NS5a region and interferon resistance. It was also reported that the NS5a region suppresses the p53 mediated apoptosis during HCV infection, which might be in association

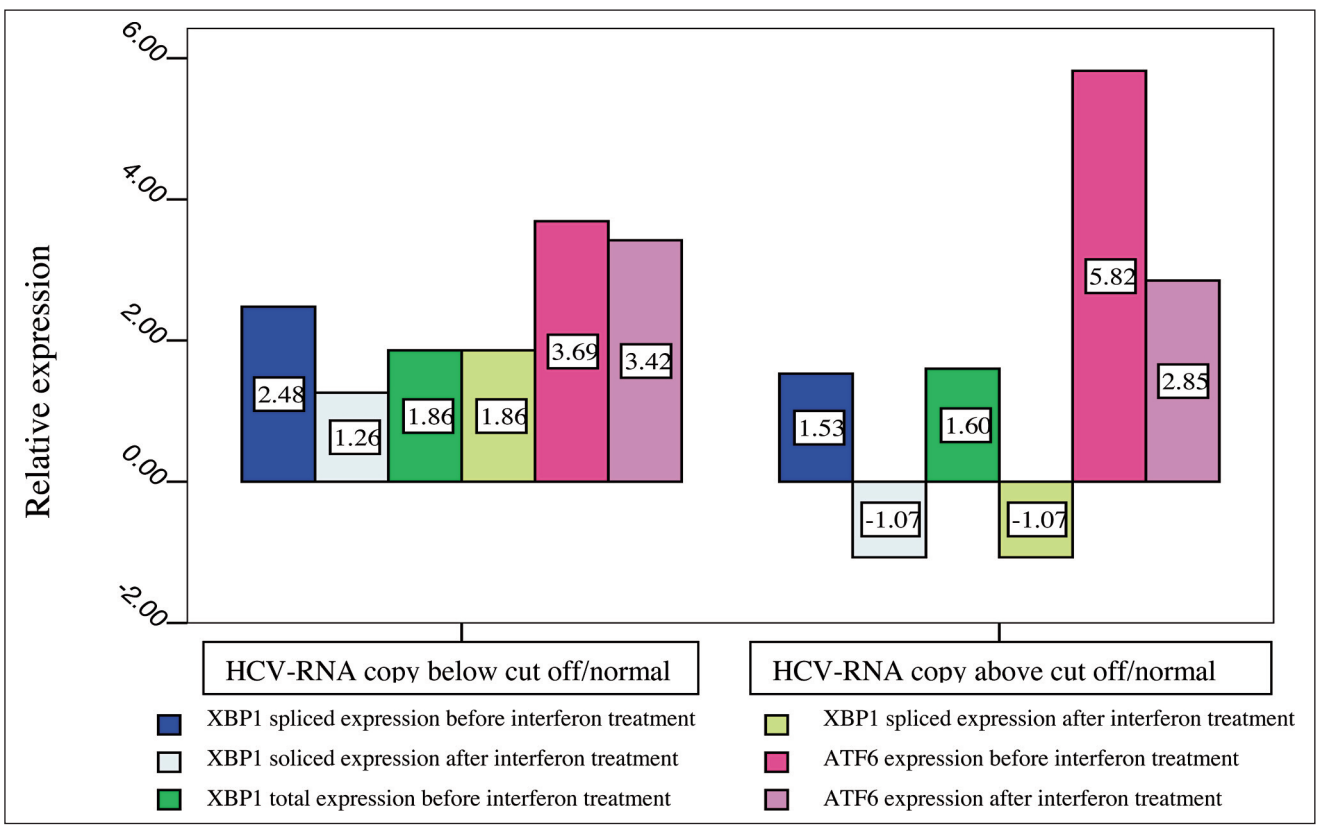

Fig. 1. Relative expression of chaperones XBP-total, XBP-spliced and ATF6. *Left side: Group2* (serum HCV-RNA PCR titre below the cut off – 8 million copy/ml). *Right side: Group1* (serum HCV-RNA PCR titre above the cut off). Chaperones are indicated with separate colours shown on the legend. Dark shade of the relevant colour indicates the relative expression before, while light one after interferon treatment

with hepatocarcinogenesis [14]. The NS5b protein is encoded in the most stable part of the genome. Structural proteins are located on the aminoterminal end, while enzyme proteins are on the carboxyterminal end of the polyprotein. Non-structural proteins, including NS3, NS4 and NS5A/B, make up a ribonucleoprotein replication complex associated with the endoplasmic reticulum (ER) membrane [12]. During their synthesis the ectodomains of HCV glycoproteins are targeted to the lumen of the endoplasmic reticulum (ER), where they are modified [15]. During polyprotein maturation, glycans promote glycoprotein folding either directly, by increasing the solubility of a protein, or indirectly, by interacting with ER chaperones. Mutated E1 proteins interfere with the folding of E2, leading to misfolding of an E2 domain that aggregates with E1 [16]. *Calnexin (CNX)* interacts with HCV glycoproteins and this chaperone has been shown to be involved in the assembly of the noncovalent E1E2 heterodimer [7]. It appears that the co precipitation of mutant E1 proteins with *calnexin* depends on the number of glycans present on the proteins and this may account for the low level of proper folding of some mutant E1 proteins. Furthermore a mutation at position N1 and predominantly at position N4 dramatically reduced the efficiency of the formation of noncovalent E1E2 complexes [16]. HCV nonstructural proteins are localized to the ER membrane, where they form a ribonucleoprotein (RNP) complex, that engages in the replication of the viral genome, an event necessary for establishing the infectious process in hepatocytes. The association of the replication complex with the ER membrane induces ER stress. This stress elicits the activation of intracellular signalling pathways from the ER to the nucleus [12]. The ER is sensitive to a variety of cellular stresses, including the alteration of Calcium homeostasis and inhibition of protein glycosylation [17]. These stresses inhibit protein folding in the ER, resulting in the accumulation of misfolded proteins in the organelle. Cells can respond to ER stress by activating two functionally separate signalling pathways, the unfolded protein response (UPR) and the ER overload response (EOR) [18]. HCV proteins induce both of these signalling pathways. The HCV protein NS5A stimulates EOR after disturbing intracellular calcium levels, leading to the activation of NF-κB and STAT-3 [19]. The HCV structural protein E2 can accumulate in the ER as misfolded aggregates inducing UPR [12]. *ATF6* is a transmembrane protein localized to the ER; the protein is regulated post-translationally by ER stress. Upon ER stress, *ATF6* is cleaved at the cytoplasmic face of the ER membrane and the resulting 50-kDa N-terminal domain, pATF6_(N) is translocated to the nucleus, where it transcriptionally activates ER chaperones whose promoters harbour several ATF6 binding sites and lead to the induction of *GRP78,* a major target of the UPR. Therefore the cap-independent translation directed by the HCV-IRES and GRP78-IRES is stimulated [12] Overexpression of *GRP78* delays the onset of apoptosis induced by unfolded proteins [20] *ATF6* also initiates the transcriptional induction of ER chaperone and *XBP1* genes in an ERSE-dependent manner [21]. *XBP1* mRNA is spliced by IRE1, an ER stress transducer. *XBP1* is a transcription factor that activates the mammalian UPR through ERSE sequences, similarly to ATF6. The HCV replication seems to alter the typical course of the UPR signalling pathway to prolong its survival in hepatocytes [12]. Lee et al. [22] reported that HCV E2 protein blocks apoptosis induced by HCV infection and the host immune system, through overproduction of *GRP94:* the activation of the well-known antiapoptotic NF-kappaB was mediated by HCV-E2-induced expression of the molecular chaperone *glucose-regulated protein 94 (GRP94)*. Therefore HCV E2 plays an important role in persistent HCV infection. Hepatitis C virus (HCV) non-structural protein 5A (NS5A) is a component of a viral replicase and is well known to modulate the functions of several host proteins. A report from Okamoto et al. [23] suggests that NS5A specifically interacts with FKBP8, a member of the FK506-binding protein family, but it does not interact with other homologous immunophilins. Since FK-506 (tacrolimus, Prograf) is a basic immunosuppressant agent used in liver transplantation, it is a fact of interest that immunoprecipitation analyses revealed that FKBP8 forms a complex with *HSP90* and NS5A. They found that treatment of HCV replicon cells with geldanamycin, an inhibitor of Hsp90, suppressed RNA replication in a dose-dependent manner. In their later studies Okamoto et al. [24] reported that examination of individual fields of the replicon cells by both fluorescence microscopy and electron microscopy revealed that FKBP8 is partially co-localized with NS5A in the cytoplasmic structure known as the membranous web. These results suggest that a specific interaction of NS5A with FKBP8 in the cytoplasmic compartment plays a crucial role in the replication of HCV.

In our study we found a significant upregulation of chaperons in the hepatocytes infected with hepatitis C virus. Patients whose serum contained a high (more than 8.78 million copy/ml) RNA titre after OLT showed an increased *ATF6* upregulation. After one year of interferon treatment the upregulation was reduced in all chaperones, and in some cases *(XBP and HSP27)* downregulation occurred. The prolonged and severe ER-stress might cause apoptosis via the activation of *C/EB1 homologous protein* (CHOP) or *JUN NH_2-terminal kinase (JNK),* or caspase 12, or by calcium depletion occurring in the endoplasmic reticulum during ER stress [25, 26]. On the other hand the deliberation of reactive oxygen species (ROS) activate STAT3 and NFκB enzymes, which protect hepatocytes from apoptosis by promoting the expression of certain genes [12]. In accordance with the literature [27] our results suggest that hepatitis C might induce apoptosis through ER-stress, however it could also limit the intensity of hepatocyte apoptosis. The theory – liver cells exposed to a high C viral load, had a lower

chance to be eliminated by this pathway – might explain why liver transplanted patients with extreme RNA-PCR titres had a worse prognosis. However it must also be taken into account that other causes of post liver transplant ER-stress also exists: Emadali et al. [28] reported that the expression of certain molecular chaperones induced by ER stress, are differentially affected upon ischemic-reperfusion injury, thus suggesting that distinct ER stress responses are activated during each phase of liver transplantation. The research of the field on extended and prospective biopsy-material is planned.

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