Molecular Biological Examination Of HCV Recurrence After Liver Transplantation, With Regard To Clinical Aspects

Ph.D. Doctoral Thesis

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Introduction

Liver cirrhosis caused by Hepatitis C virus (HCV) is one of the leading indications of liver transplantation (OLT). HCV recurrence after liver transplantation is universal and results in liver cirrhosis in many cases even after OLT. Only one-third of patients achieve SVR, which is associated with a deceleration fibrosis progression. It is a significant question whether donor selection influences the progression of HCV recurrence. Organ shortage has led transplant centers to expand their criteria for the acceptance of extended criteria donors (ECD). Donors are generally considered ECD or marginal if there is an additional risk of primary non-function (PNF) or initial poor function (IPF) after the transplantation. Many of the ECD criteria are independent risk factors for HCV recurrence. Therefore, the general opinion is to avoid the use of marginal livers into HCV positive recipients. The donor selection in Hungary is very strict in comparison to other transplant centers. Due to the strict exclusion donor selection criteria, limited clinical experience is available on the use of ECD grafts in Hungary, with special regard to the HCV positive recipient population. In addition, conflicting results are available on the use of ECD livers in high risk recipients. In a study from 2007, the use of ECD graft has been shown to be an additional risk factor for poor patient survival in patients with high pretransplant MELD (model for end-stage liver disease) score. Another study reported that high risk patients (MELD score >20) have had survival benefit from ECD livers when compared with those patients without transplantation. One of our aims was to study the influence of donor-recipient matching on the results of OLT and HCV recurrence in the Hungarian liver transplanted patients.

We examined the progression of HCV recurrence from another clinical aspect. Type 2 diabetes frequently develops after OLT which increases the rate of cardiovascular morbidity and mortality and the risk of graft rejection, graft loss and infection. Risk factors for new onset diabetes after liver transplantation (NODAT) are similar to those of type 2 diabetes in immunocompetent patients (age, obesity, metabolical syndroma etc.). The use of use of immunosupressive drugs and HCV reinfection are additional risk factors of NODAT. Insulin resistance after OLT contributes adversely to fibrosis progression and to the response to antiviral therapy. Our aim was to evaluate the effect of NODAT on HCV recurrence development and progression in the Hungarian liver transplant population.

Receptors essential for HCV entry into target cells have been studied by many research groups because these proteins might become targets for new antiviral drugs. However, the majority of examinations have been done in vitro. Our knowledge of HCV entry is the following. HCV particles associate with lipoproteins in the blood and reach hepatocytes via the sinusoidal blood. The first step in of HCV entry is the interaction of the lipoprotein associated virus particles with Lectins pont. These proteins are expressed on the surface of hepatocytes and on the sinusoidal endothel. HCV than anchored by other capture receptors (glycoseaminoglycans-GAG, lipoprotein-lipase-LPL, low-density lipoprotein receptor- LDL-R) on the basolateral surface of liver cells. After this interaction, HCV contacts with high affinity receptor SR-B1 and CD81. Then, HCV migrates to the canalicular surface of the hepatocytes where claudin-1 as a co-receptor of CD81 takes part in the process. After reaching tight junctions, the virus enters the cell via endocytosis. The membrane fusion could not be finished in the lack of claudin-1 and CD81. Other two tight junction proteins, occludin and claudin-6 have also pivotal role in the HCV entry in vitro. The

exact role of the latter receptors needs to be clarified. Among the HCV receptors, claudin-6 protein expression has not been detected in human liver yet. Only 3 experiments are available on the expression of HCV receptors in human livers and only one study examined receptor expressions in liver transplanted patients. Moreover, our knowledge of the effect of antiviral therapy on HCV receptor expressions is limited too. We examined the expression of CD81, occludin, claudin-1 and claudin-6 as recognized receptors of HCV entry in liver transplanted patients by comparing to normal liver samples and post antiviral treatment biopsies. Our aim was to correlate expressional pattern of HCV receptors with viral recurrence and antiviral therapy response.

Aims

- To examine the impact of ECD livers on general complication rate, patient survival, HCV recurrence rate and on the progression of HCV reinfection in high risk patients.
- To study the prevalence of NODAT and it's association with the time of HCV recurrence and disease progression in the Hungarian liver transplant center.
- To correlate the hepatic expression of HCV receptors with HCV recurrence and therapy response.
- To study whether HCV receptor expression existing at HCV recurrence can predict SVR.
- To detect claudin-6 protein expression in human liver tissue.

Patients And Methods

The influence of donor-recipient matching on the results of OLT was examined on the following patients and with the following methods:

Data of 260 liver transplanted patients were studied retrospectively. Transplantation procedures were performed between January 2003 and September 2009. Organs were received from deceased donors. We used the following ECD criteria: age older than 60 years, donor body mass index greater than 27, intensive care unit stay longer than 3 days, high inotropic support including dopamine dose more than 10 μkg/kg/min or need for combined therapy (dopamine and norepinephrine), hypotension (>1 hour; mean blood pressure <80 mm Hg), cardiac arrest requiring cardiopulmonary resuscitation, peak serum sodium concentration greater than 156 mEq/l, serum aspartate aminotransferase or alanine aminotransferase concentration greater than 40 U/l, serum γ-glutamyl transferase concentration greater than 60 U/l, or serum bilirubin concentration greater than 17 μmol/l. A donor was considered marginal if at least two criteria were presented. In 112 cases patients received marginal liver (group BAD). In the rest of the cases (N=148) optimal livers were transplanted (group GOOD). Recipients were grouped by their MELD scores. MELD was lower than 17 in 177 patients (group GOOD) and higher than 17 in 83 patients (group BAD). So we formed four groups according to low/high risk patients received marginal or optimal livers. Graft-, patient survival, and the rate of general postoperative complications were examined in these groups.

The association between new onset diabetes and hepatitis C virus infection was examined on the following patients with the following methods:

Data of 310 liver transplanted patients were studied retrospectively. Transplantation procedures were performed between January 1995 and January 2009. Those patients were excluded from the survey who suffered from type 1 or 2 diabetes before the liver transplantation (60 patients). Patients who had impaired fasting glicemia (IFG- fasting glucose between 6,1 and 6,9 mmol/l) after the transplantation were also excluded from the study (24 patients). Finally, patients with transient hyperglicaemia (hyperglicemia normalized during the first two postoperative months) were also excluded (20 patients). New onset diabetes was defined as a fasting plasma glucose > 7 mmol/l or random plasma glucose > 11,1 mmol/l permanently beyond three months after OLT, and/or the need for sustained antidiabetic therapy (63 patients, 20,3%). The rest of the patients (N=143) belonged to the control group.

Hepatitis C virus entry receptors were examined on the following patients with the following methods:

28 HCV-positive adult liver transplanted patients were enrolled in the study. Transplantation procedures were performed between 2003 and 2007. HCV recurred in all cases. Antiviral therapy was started only in case of histologically proved hepatitis. All selected patients received a combination of PEG-IFN and ribavirin for 12 months without interruption. 11 patients (39,3%) achieved the end-of-treatment response (ETR- HCV has been undetectable in their sera at the end of therapy). 6 of these 11 patients achieved the sustained virological response (HCV RNA was undetectable in sera 24 weeks after the end of therapy). At the end of antiviral treatment, a second liver biopsy was obtained from the patients. Therefore, we had a

pre-, and posttreatment liver biopsies from all patients. 13 normal liver samples were obtained from deceased donors during the organ receiving. HCV receptor expressions were measured in these latter samples, too. Liver samples were fixed in 10% buffered formalin and embedded in paraffin. We examined claudin-1, claudin-6, CD81 and occludin expressions in the above mentioned patient groups. mRNA expressions were measured by real-time PCR, we used β-actin as a reference gene. Protein expressions were examined by immunohistochemistry with Benchmark XT automatic immunostainer, using kvantitative (morphometry) analysis.

Statistical analysis:

We used SPSS 15. version (SPSS, Inc., Chicago, Ill) for the statistical analysis. Student's *t*-test, ANOVA test with Scheefe and Bonferroni posthoc tests and Mann-Whitney *U*-test were used for uni-, multivariate analysis, after examination population homogeneity of the variables (Levene test). The connections between continuous variables were evaluated by correlation analysis, using Pearson correlation coefficient. Cumulative graft- and patient survivals were examined by Kaplan-Meier analysis. The differences were considered to be significant when $P < 0,05$.

Results

The influence of donor-recipient matching on the results of OLT:

The 1-, 3- and 5- year patient survival rates were 93%, 86%, and 83% in group G/G; 84%, 79%, and 75,5% in group B/G; 82%, 79%, and 72% in group G/B; and 83%, 83% and 83% in group B/B, respectively. There were no difference in age, sex, cold and warm ischemic time, operation time and indication for OLT in the studied groups. IPF occurred more frequently in group G/G compared with group B/B and G/B. The rate of postoperative complications (infections, bleeding and kidney failure) were higher among high risk patients in comparison to recipients with MELD score lower than 17. The use of ECD graft has been shown to be additional risk factor of these complications. High MELD score and ECD grafts did not influence the severity of viraemia during the first six postoperative months or the histology activity index (HAI) of the liver biopsy obtained at HCV recurrence. There was no difference in the time of viral recurrence between the examined groups.

The association between new onset diabetes and hepatitis C virus infection:

In HCV-negative recipients the rate of NODAT was 15,5%, while in the HCV positive group new onset diabetes occurred in almost every second (55,8%) patient. In the control group, one-third of the patients had at least a 10-times increase in HCV viral load after the transplantation from baseline level. In the NODAT group this rapid viral replication rate could be seen in the 53% of patients. In addition, HCV recurred earlier in NODAT patients, therefore new onset diabetes predicted HCV recurrence. HCV recurred during the first three postoperative months only in the NODAT group. NODAT was developed in 80% of patients with early (within 5 months)

viral recurrence. Histology activity index and fibrosis score of liver biopsies obtained at HCV recurrence did not differ between the examined groups. However, after the one-year-antiviral-therapy indicated by HCV recurrence, HAI-, and fibrosis scores were significantly higher in NODAT patients than in seropositive but not diabetic patients.

Examination of hepatitis C virus entry receptors:

We have found a positive correlation between claudin-1 protein expression and fibrosis score. We have grouped the biopsies by the median of the fibrosis score . We found that in liver biopsies above the median fibrosis, claudin-1 protein expressions increased in comparison to claudin-1 levels in biopsies with milder fibrosis (below median). Claudin-1 expression was intensive on bile ducts in all liver sample. The immunostaining could be detected both the sinusoidal (basolateral) and canalicular (apical) surface of hepatocytes but claudin-1 expressed mainly on the canalicular site.

Claudin-1 and claudin-6 protein levels significantly increased while CD81 protein expression declined at the time of HCV recurrence in comparison to normal liver. Claudin-1 mRNA and protein expressions were higher at HCV recurrence when compared with healthy donor samples. Claudin-6 mRNA expressions declined while CD81 mRNA levels did not change considerably in HCV recurrence samples. Occludin mRNA and protein levels did not differ significantly between HCV recurrence and normal liver biopsies.

Claudin-6 and occludin expression, was mainly detectable on the apical surface of the hepatocytes. Low CLDN-6 and occludin staining was observed on the sinusoidal site as well. CD81 expression was mainly on the sinusoidal surface of liver cells, there was only a minimal staining on the canalicular membrane of hepatocytes. Occludin stained intensively, while

claudin-6 immunostaining was mild on the luminal site of bile ducts. CD81 expression was completely lack on the bile ducts.

CLDN-6 protein expression was higher at the time of HCV recurrence in patients who achieved SVR later than in those who were non-responders. Claudin-6 and occludin protein expressions have significantly declined by the end of the therapy only in SVR patients. In non-responders interferon treatment had no significant effect on claudin-6 and occludin levels. Interferon therapy had no impact on claudin-1 and CD81 protein expressions or on mRNA expression of all the examined HCV receptors.

Claudin-6 protein expression had a strong correlation with HCV viral load in SVR patients. This correlation could not be observed in non-responders. Occludin protein expression correlated with HAI and this association was independent of the fact whether the patient achieved SVR or not.

Conclusions

- Transplantation of a marginal liver to a high risk patient is associated with increased rate of early postoperative complications and has an adverse effect on patient survival in the early postoperative period.
- New onset diabetes is frequently associated with HCV recurrence and accelerates the progression of recurrence.
- Claudin-6 is expressed in human liver.
- Claudin-1, claudin-6 and occludin are expressed mainly on the apical, while CD81 is expressed almost only on the sinusoidal surface of hepatocytes both in HCV seronegative and in seropositive liver tissue.
- Increased HCV receptor expressions on cell surface are not essential for the development of HCV recurrence.
- Antiviral therapy has no significant effect on claudin-1 and CD81 expression on the hepatocyta surface in liver transplanted patients.
- Increased claudin-6 protein expression at the beginning of interferon therapy might predict SVR.
- Claudin-6 and occludin protein levels have declined by the end of antiviral therapy only in SVR patients.
- Claudin-1 protein expression correlates with the progression of liver fibrosis.
- Claudin-6 protein expression is strongly correlates with viral load, but only in SVR patients. Occludin protein expression correlates with HAI and this association was independent of therapy response.

Publications

I. Publications related to the dissertation:

1. Nemes B, Gelley F, Zádori G, Piros L, Perneczky J, Kóbori L, Fehérvári I, Görög D. Outcome of Liver Transplantation Based on Donor Graft Quality and Recipient Status. Transpl Proc 2010; 42: 2327-2330. IF: 0.993
2. Gelley F, Zadori G, Firneisz G, Wagner L, Fehervari I, Gerlei Z, Fazakas J, Papai S, Lengyel G, Sarvary E, Nemes B. Relationship between hepatitis C virus recurrence and de novo diabetes after liver transplantation: the Hungarian experience. Transpl Proc 2011; 43: 1281-1282. IF: 0.993
3. Zádori G, Gelley F, Törzsök P, Sárváry E, Doros A, Deák AP, Nagy P, Schaff Zs, Kiss A, Nemes B. Examination of claudin-1 expression in patients undergoing liver transplantation owing to hepatitis C virus cirrhosis. Transpl Proc 2011; 43: 1267-1271. IF: 0.993

II. Publications not related to the dissertation:

1. Nemes B, Gelley F, Piros L, Zádori G, Görög D, Fehérvári I, Kóbori L, Sárváry E, Nagy P, Kiss A, Doros A. The impact of Milan criteria on liver transplantation for hepatocellular carcinoma: First 15 years' experience of the Hungarian Liver Transplant Program. Transpl Proc 2011; 43: 1272-1274. IF: 0.993
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