General and disease-specific mechanistic therapy approaches for optimization of liver transplantation

PhD thesis

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1. INTRODUCTION

Liver transplantation (LTx) is a viable treatment option for acute liver failure and various end-stage liver diseases including malignancies like hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCA). The present work focuses on the development of accompanying mechanistic therapies that may be eligible for optimization of this lifesaving surgical procedure. The studies are divided into general optimization approaches of LTx and disease-specific mechanistic experiments aiming to identify suitable targets in order to improve LTx for unresectable CCA patients according to the multimodal therapy concept of the Mayo protocol.

In the general optimization approach experiments we sought to minimize liver preservation injury and improve microcirculation in implanted liver grafts by modification of a histidine-tryptophan-ketoglutarate (HTK)-based preservation solution. Specifically, we were interested in the role chloride as various *in vitro* studies of cold-induced cell injury have revealed detrimental effects of extracellular chloride on cold-stored isolated rat hepatocytes while its influence on endothelial cells is beneficial. To determine which of these effects is predominant *in vivo*, we tested both a chloride-poor variant of a new HTK-based preservation solution and a chloride-containing variant in a rat liver transplantation model. In addition, we tested the effect of the pleiotropic substance erythropoietin (EPO) on liver regeneration/donor liver growth and hepatocyte apoptosis (programmed cell death) in the setting of partial liver transplantation (pLTx).

In the disease-specific experiments we focused on CCA. Sole LTx for unresectable CCA is often associated with early disease relapse and limited overall survival. However, a small percentage of patients have achieved prolonged survival after LTx, suggesting that (neo)adjuvant approaches might improve the clinical outcome. Thus, a multimodal therapy protocol was developed at the Mayo Clinic, Rochester, Minnesota, USA employing pre-LTx external-beam irradiation, chemotherapy, and iridium brachytherapy for patients with unresectable CCA above the cystic duct and without extrahepatic metastases. After pretreatment and before LTx, patients undergo an exploratory laparotomy to exclude metastatic disease. The Mayo protocol has been proven to be quite successful for the treatment of patients with unresectable early-stage CCA. However, employing the conventional chemotherapeutic agents fluorouracil and capecitabine, this protocol does not consider new mechanistic findings on CCA tumor biology and, thus, might be improvable by the implementation of „targeted chemotherapy". The CCA-specific LTx optimization experiments thus aim to identify mechanistic processes underlying the pronounced resistance to apoptotic cell death characteristic for CCA cells. Specifically, we further examined the mechanisms whereby platelet-derived growth factor BB (PDGF-BB)/Hedgehog (Hh) signaling mediates apoptosis resistance in CCA, revealing a pivotal role for the cell division regulating serine/threonine kinase polo-like kinase 2 (PLK2). Based on these findings, new mechanistic therapy approaches were tested.

2. OBJECTIVES

The aims of the present studies were:

1. Optimization of a modified HTK-based preservation solution focusing on chloride-dependent effects on liver preservation injury and microcirculation after LTx.
2. Investigation of the impact of adjuvant administered EPO on liver regeneration/donor liver growth and hepatocyte apoptosis in the setting of pLTx.
3. Examination of the role of myofibroblast (MFB)-to-CCA cell paracrine signaling for CCA apoptosis resistance in the context of PDGF-BB/Hh co-activation networks.
4. Exploration of anti-apoptotic effects mediated by Hh/PLK signaling crosstalk.
5. Based on the observations of 3) and 4), the objectives of subsequent studies were to test whether targeting platelet-derived growth factor receptor (PDGFR)-β, Hh or PLK signaling would be therapeutic in CCA and, thus, might be a suitable (neo)adjuvant therapy to optimize the Mayo LTx protocol for CCA patients.

3. METHODS

Preservation solution/chloride study. The study, which was carried out in a blinded fashion with 7/8 rats per group, was divided into 2 parts: (1) comparison of survival in 3 series under different conditions (different microsurgeons, rat strains, cold and warm ischemia times) and (2) assessment of microcirculation (30-90 minutes after reperfusion), laboratory data, bile production and histology after LTx with a chloride-poor and a chloride-containing variant of a new HTK-based preservation solution.

Erythropoietin study. Rats were treated with EPO or heat-inactivated EPO-vehicles. Animals underwent 30% partial pLTx. Serum, bile and liver samples were taken to investigate liver function, liver-to-body weight ratio (LBWR), hepatocyte-proliferation (Ki-67) and apoptosis (terminal deoxynucleotide tranferase-mediated dUTP nick-end labeling-assay). Gene expression was assessed by an in-house cDNA array and quantitative real-time polymerase chain reaction. Finally, overall survival was assessed.

MFB-derived PDGF-BB/Hh signaling study. We employed human KMCH-1, KMBC, HuCCT-1, TFK-1 and Mz-ChA-1 CCA cells as well as human primary hepatic stellate and myofibroblastic LX-2 cells for these studies. *In vivo* experiments were conducted using a syngeneic rat orthotopic CCA model.

Hh signaling/PLK 2 study. We employed 50 human CCA samples (25 intrahepatic and 25 extrahepatic CCA specimens) as well as human KMCH-1, Mz-CHA-1 and HUCCT-1 CCA cells for these studies. *In vivo* experiments again were conducted using the syngeneic rat orthotopic CCA model.

4. RESULTS

4.1. General optimization approaches for LTx

4.1.1. Preservation solution/chloride study

In each of the survival experiments, a strong tendency toward prolonged survival was observed with the new chloride-containing solution (50% versus 12.5%, 75% versus 37.5%, and 100% versus 71.4% [chloride-containing vs. chloride-poor], overall $P < 0.05$). Additionally, the sinusoidal perfusion rates (83.9% ± 4.0% versus 69.2% ± 10.8%, $P < 0.01$) and the red blood cell velocities in sinusoids (147.7 ± 26.7 versus 115.5 ± 26.0 μm/second, $P < 0.05$) and in postsinusoidal venules (332.4 ± 87.3 versus 205.5 ± 53.5 μm /second, $P < 0.01$) were clearly higher with chloride. Moreover, the serum activities of liver enzymes were slightly reduced (not significantly) and bile production was significantly increased.

4.1.2. Erythropoietin study

EPO led to improved liver regeneration as shown by an increased LBWR and Ki-67 index in pretreated donor rats as well as rats after pLTx. EPO treatment induced the pro-regenerative mediator *c-jun* and the anti-apoptotic gene *Bcl-X_L*, which was accompanied by a decreased apoptosis rate. In addition, EPO-treated rats showed a significantly improved 28-day survival after pLTx (88% vs. 38%).

4.2. Optimization of LTx for cholangiocarcinoma

4.2.1. MFB-derived PDGF-BB/Hh signaling study

Co-culturing CCA cells with myofibroblastic human primary hepatic stellate cells or LX-2 cells significantly decreased tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in CCA cells, a cytoprotective effect abrogated by neutralizing PDGF-BB antiserum. Cytoprotection by PDGF-BB was dependent upon Hh signaling, because it was abolished by the smoothened (SMO; the transducer of Hh signaling) inhibitor cyclopamine. PDGF-BB-induced cyclic adenosine monophosphate–dependent protein kinase-dependent trafficking of SMO to the plasma membrane, resulting in glioma-associated oncogene (GLI)2 nuclear translocation and activation of a consensus GLI reporter gene-based luciferase assay. A genome-wide messenger RNA expression analysis identified 67 target genes to be commonly up- (50 genes) or down-regulated (17 genes) by both sonic hedgehog (SHH) and PDGF-BB in a cyclopamine-dependent manner in CCA cells. Finally, in a rodent CCA in vivo model, cyclopamine and imatinib administration increased apoptosis in CCA cells, resulting in tumor suppression.

4.2.2. Hh signaling/PLK 2 study

In human samples, PLK1/2/3-immunoreactive cancer cells were present in the preponderance of intra- and extrahepatic CCA specimens. Inhibition of Hh signaling by cyclopamine reduced PLK2, but not PLK1 or PLK3, messenger RNA and protein expression in vehicle-treated and SHH-treated CCA cells, confirming our previous microarray study. PLK2 regulation by Hh signaling appears to be direct, because the Hh transcription factors GLI1 and GLI2 bind to the PLK2 promotor. Moreover, inhibition of PLK2 by the PLK inhibitor BI 6727 (volasertib) or PLK2 knockdown was pro-apoptotic in CCA cells. BI 6727 administration or PLK2 knockdown decreased cellular protein levels of antiapoptotic myeloid cell leukemia 1 (Mcl-1), an effect reversed by the proteasome inhibitor, MG-132. Finally, BI 6727 administration reduced Mcl-1 protein expression in CCA cells, resulting in CCA cell apoptosis and tumor suppression *in vivo*.

5. CONCLUSIONS

The observations from the studies presented suggest that:

1. *In vivo,* beneficial effects of chloride-containing preservation solutions on the endothelium/microcirculation exceed chloride-dependent hepatocyte injury occurring during cold storage. Therefore, HTK-based and possibly other preservation solutions for liver (and vessel) grafts should contain chloride.
2. Erythropoietin treatment significantly improves liver regeneration and survival after pLTx, in part, by upregulation of the pro-regenerative mediator *c-jun* as well as the anti-apoptotic gene *Bcl-X_L*. Thus, EPO application may represent a promising adjuvant strategy to optimize the clinical outcome especially after sLTx and LDLT.
3. MFB-derived PDGF-BB protects CCA cells from TRAIL cytotoxicity by a Hh signaling–dependent process. Targeting PDGFR-β and/or Hh signaling sensitizes these tumor cells to apoptotic cell death. Therefore, these results might have therapeutical implications for the pretreatment of patients awaiting LTx for CCA.
4. PLK2 appears to be a pivotal mediator of Hh survival signaling in CCA cells. Targeting the Hh/PLK signaling co-activation network also sensitizes CCA cells to apoptosis. Thus, besides Hh inhibitors, PLK inhibitors might be of therapeutic value for the multimodal treatment of human CCA including the Mayo LTx protocol

6. PUBLICATIONS OF THE CANDIDATE

6.1. Publications related to the PhD thesis

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(Impact-factor 2006: 4.0; * joint 1st authorship)

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