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Moesin functionality in hypothermic liver preservation injury ☆

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ABSTRACT

The objective of this study was to determine how expression and functionality of the cytoskeletal linker protein moesin is involved in hepatic hypothermic preservation injury. Mouse livers were cold stored in University of Wisconsin (UW) solution and reperfused on an isolated perfused liver (IPL) device for one hour. Human hepatocytes (HepG2) and human or murine sinusoidal endothelial cells (SECs) were cold stored and rewarmed to induce hypothermic preservation injury. The cells were transfected with: wild type moesin, an siRNA duplex specific for moesin, and the moesin mutants T558D and T558A. Tissue and cell moesin expression and its binding to actin were determined by Western blot. Liver IPL functional outcomes deteriorated proportional to the length of cold storage, which correlated with moesin disassociation from the actin cytoskeleton. Cell viability (LDH and WST-8) in the cell models progressively declined with increasing preservation time, which also correlated with moesin disassociation. Transfection of a moesin containing plasmid or an siRNA duplex specific for moesin into HepG2 cells resulted in increased and decreased moesin expression, respectively. Overexpression of moesin protected while moesin knock-down potentiated preservation injury in the HepG2 cell model. Hepatocytes expressing the T558A (inactive) and T558D (active) moesin binding mutants demonstrated significantly more and less preservation injury, respectively. Cold storage time dependently caused hepatocyte detachment from the matrix and cell death, which was prevented by the T558D active moesin mutation. In conclusion, moesin is causally involved in hypothermic liver cell preservation injury through control of its active binding molecular functionality.

Introduction

Liver transplantation continues to be the treatment of choice for patients with end stage liver disease. The limiting factor is the availability of suitable liver grafts. In 2012, 6256 livers were transplanted in the US out of a pool of 16,446 (38%). Conversely, 62% or over 10,000 patients were left on the waiting list for a graft [9]. It has recently been shown that using liver grafts from extended criteria organ donors decreases the wait time without adversely affecting post-transplant survival [23]. However, as the quality of donor livers continues to decrease because we are selecting donors that were once considered unusable, significant improvements in organ preservation will be required to maintain adequate function in these grafts.

The amount of cold exposure during hypothermic liver preservation is directly related to the amount of preservation injury experienced by the graft after transplantation [7]. In fact, cold ischemia time is one of the single most important risk factor for liver preservation injury [22] and it causes later graft injury even when normal function returns shortly after liver transplantation [1]. Cold ischemia, which is proportional to graft preservation injury after transplantation [20,26], significantly sensitizes the hepatocyte cell membrane to rewarming injury. Furthermore, cold exposure during hypothermic preservation of livers causes sinusoidal endothelial cells (SEC) to die and detach from the peri-sinusoidal matrix plate. This exposes underlying tissue factor, which activates coagulation and promotes thrombosis leading to low flow and further ischemia at reperfusion [2,17]. Detachment and subsequent apoptotic cell death in hepatocytes is also dependent on β1 integrin failure with the cell membrane [19]. Therefore, a

Abbreviations: ERM, ezrin radixin moesin; IPL, isolated perfused liver; UW, University of Wisconsin solution; SEC, sinusoidal endothelial cell; LDH, lactate dehydrogenase; FERM, protein 4.1, ezrin, radixin, moesin; LPA, lysophosphatidic acid; PKC, protein kinase C; ROK, Rho kinase.

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reasonable strategy to protect liver grafts from cold ischemic injury during organ preservation is to understand the fundamental mechanisms of how the cell membrane and underlying structures are injured during hypothermic preservation in order to identify rational therapeutic targets.

Cell membrane ultrastructure (microvilli), cell membrane organization, and cell polarity are developed and maintained, in large part, by the sub-lamellar cytoskeletal system [15]. This system consists mostly of fodrin and the band 4.1 superfamily of proteins, which consists of protein 4.1, ezrin, radixin, and moesin (FERM). These proteins all have a FERM domain at the amino terminus that binds a wide variety of membrane associated target proteins and an actin binding domain (ABD) at the carboxy terminus that links to actin [4]. These linker proteins help support ultrastructural components of epithelial cells, which are important to the function of highly epithelialized organs like the liver. While most cells produce all of these proteins, the cells of the liver express moesin and radixin but not ezrin [5].

The functional significance of ERM proteins in hypothermic preservation injury is suggested by a series of studies of ezrin expression and functionality in renal tubular epithelium [16,24,25]. Ezrin disassociates from the actin cytoskeleton proportional to the degree of preservation injury in renal tubules [16]. Ezrin is protective in preservation injury by virtue of its degree of expression and its binding configuration. This implicates a role of the threonine-567 (T567) specific phosphorylation site of ezrin [24,25] since this site controls ezrin binding. Liver tissue does not readily express ezrin but it does display membrane injury with cold ischemia that is similar in kidneys. Therefore, an analogous hepatic ERM mechanism of cell membrane injury during cold storage preservation likely exists involving moesin and radixin in the liver. The role of moesin in hypothermic liver preservation injury was the specific focus of this study. Our hypothesis is that moesin serves to protect hepatocytes during hypothermic liver preservation injury by its membrane linker functionality, which facilitates cell adhesion and viability. The following experiments were designed to test this hypothesis.

Materials and methods

Mouse liver preservation model

All studies involving animals were first approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (IACUC). VCU is an AAALAC accredited institution. Mice were anesthetized with isofluorane and the liver was quickly removed after in situ flushing with cold UW solution through the portal vein. Livers were cold stored in UW solution at 2–4 °C on ice in a refrigerator for 0, 24, or 48 h. After cold storage, the livers were reperfused with Krebs buffer on an IPL for an additional 60 min with oxygenation to simulate reperfusion at transplantation. This was done as previously described in detail [14]. The purpose was to demonstrate a correlation between cold storage time and preservation injury outcomes in the liver after reperfusion for later comparisons with tissue moesin binding. The measured endpoints to assess liver preservation injury were bile production, vascular resistance, and lactate dehydrogenase (LDH) release into the IPL buffer. In 3 additional mice, sinusoidal endothelial cell attachment was determined by the in situ trypan blue test as previously described [14]. In a separate group of mouse livers, tissue samples were obtained before cold storage, after 6 or 24 h of cold storage, and after 24 h of cold storage and 1 h of normothermic oxygenated reperfusion on the IPL. The tissue was extracted and assayed for moesin in its unbound and its cytoskeletal bound state by Western blot.

Cell preservation model

A cell model of hypothermic liver preservation injury was developed using both sinusoidal endothelial cells and hepatocytes. Mouse sinusoidal endothelial cells (mSEC, a gift from Dr. Vijay Shah, Mayo Clinic), human sinusoidal endothelial cells (hSEC, ScienCell, Carlesbad, CA), and human hepatocytes (HepG2 cells, ATCC, Manassas, VA) were grown separately to 80% confluence in 96-well plates in DMEM with 5% fetal calf serum. To induce hypothermic preservation injury, the plates were placed in an air tight chamber (Tupperware), which was purged with nitrogen for 5 min and placed in a refrigerator on ice for 4–48 h. Following cold storage ischemia, the cells were rewarmed and reoxygenated (to simulate reperfusion at transplantation) by placing the plates back into the 37 °C CO_2 incubator for 1 h. During the rewarming period, some wells contained 10 μl of WST-8 for the detection of cell viability and mitochondrial metabolism. After 60 min, the plates were read (450 nM) to assess WST-8 oxidation. LDH release was determined with a CytoTox 96 assay kit to assess membrane integrity. Some cells that were plated in 6 well plates were extracted with a differential moesin extraction buffer after reperfusion to measure moesin disassociation as previously described in detail for ezrin [16].

Materials

Prk7 Moesin-YFP, an expression plasmid containing mouse wild-type Moesin cDNA, was a gift from Dr. Kodi S. Ravichandran, University of Virginia School of Medicine, Charlottesville, VA. The human sinusoidal endothelial cell line hSEC was purchased from American Type Culture Collection (ATCC). CytoTox 96 non-radioactive cytoxicity assay kits (LDH based) were ordered from Promega Corp. (Madison, WI). Cell proliferation reagent WST-8 was ordered from Cayman Chemical (Ann Arbor, Michigan).

Site-directed mutagenesis

To substitute the COOH-terminal threonine 558 with an alanine (T558A) or an aspartate (T558D), site-directed mutation was done with the QuikChange®II site-directed mutagenesis kit (Agilent technologies, Santa Clara CA). The oligonucleotides used to introduce the T558A and T558D mutations in Moesin were: Mutation T to A, forward primer: CCGAGACAAATACAAG**GCC**CTGCGCCAGATC CGGC, reverse primer: GCCGGATCTGGCGCAG**GGC**CTTGTATTTGTCT CGg. Mutation T to D, forward primer: CCGAGACAAATACAAG**GAC**C TGCGCCAGATCCGG, reverse primer: CCGGATCTGGCGCAG**GTC**CTT GTATTTGTCTCGG. Samples were denatured at 95 °C for 30 s and then subjected to 12 cycles of amplification (95 °C, 30 s; 55 °C, 1 min; 68 °C, 12 min) for the T558D and T558A mutations, respectively. The mutations were confirmed by DNA sequencing.

Stably transfected cell lines

Stably transfected HepG2-Moesin T558, HepG2-Moesin T558A, and HepG2-Moesin T558D cells were created by transfecting HepG2 cells with the Prk7 Moesin-YFP, Prk7 Moesin-T558A-YFP, or the Prk7 Moesin-T558D-YFP plasmid. Cells were purified by flow cytometry to create stable cell lines.

Moesin knockdown

Cells at 80% confluence were transfected using Lipofectamine 2000 Reagent with a Stealth RNAi (Invitrogen) specific for mouse moesin for 48 h and validated by Western blot.

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