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The role of Tec kinase signaling pathways in the development of Mallory Denk Bodies in balloon cells in alcoholic hepatitis

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ABSTRACT

Several research strategies have been used to study the pathogenesis of alcoholic hepatitis (AH). These strategies have shown that various signaling pathways are the target of alcohol in liver cells. However, few have provided specific mechanisms associated with Mallory-Denk Bodies (MDBs) formed in Balloon cells in AH. The formation of MDBs in these hepatocytes is an indication that the mechanisms of protein quality control have failed. The MDB is the result of aggregation and accumulation of proteins in the cytoplasm of balloon degenerated liver cells. To understand the mechanisms that failed to degrade and remove proteins in the hepatocyte from patients suffering from alcoholic hepatitis, we investigated the pathways that showed significant up regulation in the AH liver biopsies compared to normal control livers (Liu et al., 2015). Analysis of genomic profiles of AH liver biopsies and control livers by RNA-seq revealed different pathways that were up regulated significantly. In this study, the focus was on Tec kinase signaling pathways and the genes that significantly interrupt this pathway. Quantitative PCR and immunofluorescence staining results, indicated that several genes and proteins are significantly over expressed in the livers of AH patients that affect the Tec kinase signaling to PI3K which leads to activation of Akt and its downstream effectors.

1. Introduction

Liver injury from alcohol abuse involves both parenchymal and non-parenchymal cells including resident and recruited immune cells that contribute to liver damage and inflammation (Friedman, 2000). This can cause balloon hepatocytes and Mallory-Denk Body (MDB) formation. MDBs are composed of intracellular aggregations of misfolded proteins (increased β sheets) in ballooned hepatocytes (Kachi et al., 1993). They consist of abnormally phosphorylated, ubiquitinated, and cross-linked keratins 8 and 18 (K8/K18) and non-keratin components (Haybaeck et al., 2012). Three mechanisms of MDB formation (epigenetic mechanisms, shift from the 26S proteasome to the immunoproteasome and activation of Toll-like signaling) have recently been identified (French et al., 2010). The combination of these three mechanisms forms MDBs. Multiple membrane receptors that are involved in a variety of downstream responses including Ca^{2+} influx, proliferation, differentiation, motility apoptosis, gene expression, actin reorganization and adhesion/migration (Chau et al., 2002) can affect these mechanisms. However, the detailed molecular events involved in liver MDB formation, especially in human liver disease development, remains undetermined.

The Tec tyrosine kinases are one of the largest families of none-receptor tyrosine kinases (nRTK) that catalyze phosphorylation of tyrosine residue of other proteins. They are distributed in both the cytoplasm and nucleus and their role is to mediate signal transmission through activation by stimulation of membrane receptors (Middendorp et al., 2003). Tec kinase is one of the members of the Tec family. It is predominantly expressed in hematopoietic cells and hepatocytes in mammals. The function of Tec in hepatocytes is not well understood. It has been suggested that Tec kinase plays a role in hepatocyte proliferation and liver regeneration (Xu et al., 2000). Members of these kinase families function downstream of the membrane receptors including cytokine receptors, lymphocyte surface antigens, GPCR (G-Protein Coupled Receptors), receptor type PTKs, TLR or Integrin, in hematopoietic cells and transduce signals leading to calcium mobilization, altered gene expression, production of cytokines and cell proliferation.

GPCRs constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. They have three subunits: G-AlphaQ, G-Beta and GN-Gamma.

RTKs (Receptor tyrosine kinases) are the high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and

Abbreviation: MAPKs, Mitogen Activated Protein Kinases; JNK, c-Jun Kinase; PYK2, Proline-Rich Tyrosine Kinase-2; NF-κB, Nuclear Factor-KappaB; PIP3, Phosphatidylinositol-3,4,5-Triphosphate; IKKs, I-κB-Kinases; ITK, (IL-2-Inducible T-cell Kinase)/EMT/TSK; PI3K, Phosphoinositide 3-Kinase; STATs, Signal Transducer and Activator of Transcription

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hormones (Robinson et al., 2000). Receptor tyrosine kinases have been shown not only to be key regulators of normal cellular processes but also to play a critical role in the development and progression of many types of cancer (Zwick et al., 2001). PAKs (p21-Activated Protein Kinases) are a growing family of serine/threonine protein kinases, which are activated by RTKs in response to extracellular signals and regulate cell shape and motility (Tang et al., 1998). PAKs have been reported to be the regulators of diverse cellular functions, including regulation of cytoskeletal dynamics, cell survival, apoptosis, hormone signaling, and gene transcription. They are able to promote cell proliferation (Bokoch, 2003; Hofmann et al., 2004; Kumar et al., 2006; Kumar and Vadlamudi, 2002; Wells and Jones, 2010). There are six different PAKs in mammals, divided into group I (PAK1–3) and group II (PAK4–6) based on the differences in their sequence, structure, and regulatory properties (Jaffer and Chernoff, 2002; Kumar et al., 2006).

Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain that function in cell surface adhesion and signaling (Humphries, 2000). Integrin alpha 6 subunits (ITGA6) may associate with a beta 1 or beta 4 subunit to form an integrin that interacts with extracellular matrix proteins including members of the laminin family (Lee et al., 1992). Integrin alpha 6 (ITGA6) beta 4 integrin may promote tumorigenesis, while the integrin alpha 6 (ITGA6) beta 1 integrin may negatively regulate erbB2/HER2 signaling (Kumar, 1998). Alternative splicing results in multiple transcript variants.

Activation of Toll-like receptor (TLR) signaling stimulates inflammatory and proliferative pathways. It has been shown that TLR signaling activation is the key element in the pathogenesis of Mallory-Denk bodies (MDBs) in mice fed DDC (Bardag-Gorce et al., 2010). However, little is known as to how TLR signaling is regulated and functions in MDB formation during chronic liver disease development. The TLR signaling pathways are up regulated in various chronic liver diseases, which produce inflammatory cytokines and chemokines to initiate the inflammatory cascade and are involved in activation of innate immunity in AH and NASH (Petrasek et al., 2013). The first systematic study of TLR signaling pathway transcript regulation in human liver biopsies with MDB formation was published by this lab (Liu et al., 2014a, 2014b, 2014c). The results showed the up regulation of TLRs (TLR3 and TLR4) as well as transcript factors (NF-κB and IRF7, etc.) in the patient's liver. Toll-like receptor (TLR) activation results in the activation and up regulation of the protein tyrosine kinases.

Among the intracellular signaling molecules that are most crucial for innate immunity downstream of all the receptors on the cell membrane, are the cytoplasmic tyrosine kinases. These include the Src-family, the Syk family and the Tec family kinases. Previously, we have shown the over expression of the proteins such as spleen tyrosine kinase (Syk), and Protein Kinase B also known as Akt (Afifiyan et al., 2017) in the MDB forming liver cells. We have also indicated the up regulation of PI3K and mTOR in the liver cell cytoplasm in alcoholic hepatitis.

The expression of Tec tyrosine kinases and their activation downstream of multiple membrane receptors as well as the genes involved in this family, were evaluated in the liver biopsies from alcoholic hepatitis (AH) patients. This was in search of the role of protein quality control in alcoholic hepatitis pathogenesis and the mechanisms of protein quality control in alcoholic hepatitis (AH) hepatocytes. MDB formation develops in the hepatocytes in alcoholic hepatitis as a consequence of the failure of the protein quality control mechanisms to remove misfolded and damaged proteins and to prevent MDB aggresome formation within the cytoplasm of hepatocytes (French et al., 2017). Therefore, our study is focused on the identification and quantification of these proteins in the liver of patients with AH.

2. Material and methods

2.1. Liver biopsy specimens

Human formalin-fixed paraffin-embedded (FFPE) liver biopsies from patients who had alcoholic hepatitis were obtained from Harbor UCLA hospital archives as well as clinical trial funded by NIH/NIAAA grant “alcoholic hepatitis pathogenesis as determined from human liver tissue analysis” exempted as determined by the IRIS system. In all the cases, MDBs had formed in the liver. Normal livers were used for controls. The liver biopsies had been used in previous studies (French et al., 2012; Liu et al., 2014a, b). Liver biopsy sections were cut 5 μm thick. The study was carried out according to the principles of the Declaration of Helsinki and was designated as exempt by our institutional ethics review board. The data were analyzed anonymously and reported.

2.2. RNA isolation

RNA isolation of FFPE sections from human liver biopsies was performed as previously described (Afifiyan et al., 2017). Briefly, the paraffin-embedded tissue sections were submerged in xylene at room temperature for 1 h changing the xylene once after 30 min. The samples were hydrated by washing progressively for 2 min in 100%, 70%, 50% ethanol, and then pure RNase-free water. After air-drying the samples on the slides, RNA isolation was processed using the Pinpoint™ Slide RNA isolation System II (ZYMO) by adding Pinpoint™ Solution directly to the tissue section and allowing the solution to dry completely at room temperature. The embedded tissue was then removed from the slide and transferred to a micro-centrifuge tube for subsequent proteinase K digestion and RNA extraction according to the manufacturer's protocol (ZYMO). DNA-free RNA was obtained with subsequent DNase I treatment following the manufacturer's recommended protocol (ZYMO). The quality and yield of the resulting total RNAs were assessed with an absorbance reading at 260 nm (A260) using a Thermo Scientific NanoDrop™ Spectrophotometer by loading 1 μl of the extracted RNA. RNA sequencing (RNA seq).

Libraries for RNA-Seq were prepared with Nugen Ovation Human FFPE RNA-Seq Multiplex System as previously described (Liu et al., 2015). Expression pattern, function enrichment and network analysis of differentially expressed genes (DEGs) were identified using the Partek software.

2.3. Quantitative real-time PCR analysis

Synthesis of first-strand cDNAs was performed with the above mentioned total RNA (1 μg), and random hexamer primers using qScript cDNA XLT cDNA Synthesis SuperMix (Quanta Biosciences, Inc.) following instructions. Real-time PCR was performed using the Fast SYBR Green Master Mix on a StepOnePlus™ Real-time PCR System (Applied Biosystems) with a primer concentration of 300 nM. Primer sequences and the related gene Accession Number are listed in Table 1. Reaction conditions consisted of 95 °C for 20 s. followed by 40 cycles of 95 °C for 3 s. 60 °C for 30 s. Single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Human α-tubulin was used as controls to normalize the starting quantity of RNA. Quantitative values were obtained from the threshold PCR cycle number (CT) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its endogenous control level and the relative mRNA expression levels were analyzed using the ΔΔCT method. Reaction of each sample was performed in triplicate.

2.4. Immunohistochemical analysis

Formalin fixed, paraffin embedded tissue slides were immunohistochemically double stained for TEC, PAK6, GNG2, STAT2, SRC (Abcam Inc., Cambridge, MA), GNA15 (MyBioSource Inc., San Diego, CA, GNAI1 (BioSS Inc., Woburn, MA) and Ubiquitin (Millipore, Temecula, CA). TEC, PAK6, GNA15, GNG2, GNAI1, STAT2, and SRC