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Alcoholic Steatohepatitis (ASH) Causes More UPR-ER Stress than Non-Alcoholic Steatohepatitis (NASH)

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1 Introduction

ER plays an essential role in the secretory pathways and is the destination where proteins are distributed into the endo or exocytotic pathways. Assembly, folding and disulfidation of proteins occur in the ER before exposure to the extracellular space. Concentration of proteins in the ER lumen is extremely high (Stevens and Argon, 1999) and there is about 13 million secretory proteins per minute in hepatocytes (Snapp, 2005). The liver cell consumes enormous amounts of energy to perform this transportation accurately. Energy imbalance occurs for many reasons such as starvation, changes in oxidation-reduction balance, calcium level, post translational adjustments or secretory protein synthesis (Rutkowski et al., 2004). This imbalance creates a lipotoxic environment by accumulation of lipids and misfolded proteins in the ER lumen (Lake et al., 2014) and leads to the activation of BiP/GRP78 (immunoglobulin heavy chain-binding protein/glucose-regulated protein of molecular weight 78 kDa) a chaperone member of heat-shock protein 70 (Hsp70) (Wu and Kaufman, 2006). This is part of a compensatory program called “ER stress signaling” and the subsequent “Unfolded Protein Response (UPR)”. There are three determined sub-pathways involve in the UPR response, PKR-like ER protein kinase (PERK), Inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), associate with BiP in their inactive states. In severe ER stress, BiP loses its bond (Ma et al., 2002) from UPR components simultaneously. Each sub-pathway initiates a special reaction in the metabolic processes, but the immediate response starts by activation of PERK (Shi et al., 1998, Harding et al., 1999) which phosphorylates eucaryotic translation initiation factor 2 (eIF2 α). Phosphorylated eIF2 α decreases the rate of mRNA translation to attenuate general protein synthesis and also activates transcription factor 4 (ATF4) (Harding et al., 2000). BiP detachment from IRE1 α , or binding unfolded protein itself to IRE1 α (Sano and Reed, 2013), activates the IRE1 α site-specific endoribonuclease (RNase). This RNase activity splices X-box-binding protein 1 (XBP1) mRNA (Shen et al., 2001). Activated ATF6 translocates to the cis- Golgi compartment and splits up into fragments. N-terminal fragment

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The authors declare that there are no conflicts of interest.

of ATF6, along with ATF4 and spliced XBP1, migrate to the nucleus and act as active transcription factors to enhance the gene expression of proteins that augment ER protein folding capacity (Cinaroglu A et al., 2011, Tirasophon et al., 1998, Haze et al., 1999, Ye et al., 2000, Yoshida et al., 2000, Shen et al., 2001, Yoshida et al., 2001, Calton et al., 2002, Lee et al., 2002).

ER stress, under a severe insult, such as chronic alcohol consumption, activates two protein degradation pathways to preserve cell function and viability (Bernales et al., 2006). 1) The ER-assisted degradation process (ERAD) accumulates increased stress proteins, e.g. HSPs, and other components of the increased metabolic processes in the cytosol for ubiquitination and degradation by the 26S proteasome. Mallory- Denk Body (MDB) contains many ubiquitinated misfolded proteins, mostly located in ballooned hepatocytes might produce ERAD (Basaranoglu et al., 2011, Liu et al., 2014) 2) Autophagy which is mediated by lysosomal derived catalytic enzymes digestion.

Autophagy, initiated by ER stress mechanisms, varied in the current studies. one of the proposed mechanisms is related to Ca^{2+} release from ER which activates the CamKK/ AMPK-dependent pathway. This complex inhibits mTOR to induce autophagy (Høyer-Hansen et al., 2007). PERK also regulates autophagy on ATG genes via ATF-4 transcriptional effects (Harding et al., 2000). There are other cell protective pathways during ER stress that act as apoptotic suppressors. Bax-inhibitor1 (BI-1), that located in the ER membrane, provides protection during ER stress by a) regulates ER Ca^{2+} homeostasis through Bcl-2 family members and b) inhibits IRE1 α RNase activity (Xu et al., 2008).

When compensatory pathways fail after chronic and prolonged ER stress, cell dysfunction and cell death develop. Based on the longevity and severity of the stress, IRE1 α activates downstream proteins resulting in cell death and apoptosis, such as Jun-N-terminal kinase, ASK1 (apoptosis signal-regulating kinase1) and p38 MAPK (Pincus et al., 2010, Ron and Hubbard, 2008). p38 MAPK activated by IRE1 α phosphorylates and activates transcription factor CHOP (transcriptional factor C/EBP homologous protein). CHOP increases the expression of genes facilitating apoptosis (Puthalakath et al., 2007). CHOP is also one of the targets activated with ATF4 and is known as one of the most important regulators of ER stress induced apoptosis (Oyadomari and Mori, 2004, Cullinan and Diehl, 2007). Bax/Bcl-2 complex is another mediator inducing apoptosis via controlling ER Ca^{2+} release (Chae et al., 2004, Ishikawa et al., 2011). Bax also will be activated as pro-apoptotic agents by p38MAPK and JNK (Kim et al., 2006).

Chronic alcohol consumption is a well-known stressor and induces ER stress signaling / UPR. Since the mechanisms and causes that induce ASH and NASH are different, we surveyed the expressions of proteins and mediators that take part in ER stress pathway (Fig. 1) in the liver of alcoholic hepatitis and NASH.

2 Materials and Methods

Formalin-fixed paraffin embedded (FFPE) human liver biopsies from patients who were diagnosed with ASH and NASH from Harbor-UCLA hospital archived and a clinical trial

funded by NIH/NIAAA grant, i.e., “Alcoholic hepatitis pathogenesis as determined from human liver tissue analysis”(exempted as determined by the iRIS system) were compared to normal controls. In this study we studied 3-5 ASH, 3-5 NASH and 3 normal liver controls. The slides were double stained for ubiquitin plus PERK, IRE1, ATF4, ATF6 and BAX. Texas Red (Millipore, Temecula, CA) was used to detect ubiquitin. The other proteins were detected as green fluorescence by using either donkey-anti mouse or anti rabbit Alex Fluor for the secondary antibody labeled (Jackson Labs, West Grove, PA). The nuclei were stained by DAPI. The slides were all stained together at the same time to provide accurate comparisons between the different groups. We obtained the intensity of the fluorescent staining in 3 different areas on each slide with the 40x objective and 800ms standard exposure time by using a Nikon 400 fluorescent microscope. The Nikon morphometric system was used to a quantitate measurement of the fluorescent intensity. The mean, standard error and statistical differences of data achieved from the Nokia were analyzed by Graphpad statistical software. Controls vs. ASH, controls vs NASH and ASH vs. NASH were compared by unpaired t-test with $p < 0.05$. (See table 1)

3 Results

3.1 Alcoholic Steatohepatitis (ASH) causes severe liver injury due to excessive alcohol consumption. Chronic alcohol intake and exhausting cytoprotective pathways push the liver cells to lose cell function through different pathways, e.g. ERAD, which leads to MDB formation and apoptosis. The pathology of ASH, the presence of MDBs, excess fat and misfolded proteins, ballooning degeneration and degrees of fibrosis indicated a severe ER stress response. In this study the expression of PERK was significantly increased in ASH compared to the controls (Fig. 2). The expression of IRE-1 α , ATF6 and ATF4 were tending to be increased compared to the controls (Fig. 4, 5, 6). Bax, tended to be increased specifically in ASH samples with MDB formation (Fig. 7). PERK was found to be colocalized with ubiquitin in in Mallory Bodies (Fig. 3).

3.2 Non-Alcoholic Steatohepatitis (NASH) is one of the causes of fatty liver, occurring when fat is increased and stored in the liver due to obesity, metabolic syndrome or insulin resistance. Steatosis is usually combined with ballooning degeneration, MDB formation, inflammation and fibrosis. The progression of NASH to cirrhosis is slower than ASH due to presence of alcohol abuse. In NASH the expression of the first line ER stress regulators PERK and IRE-1 were significantly increased in comparison to controls (Fig. 2). ATF6 and ATF4 expressions both were decreased in NASH compared to controls (Fig. 4, 5).

3.3 ASH vs. NASH The expression of PERK and IRE-1 tended to be increased in NASH compared to ASH (Fig. 2, 4). The level of ATF4, ATF6 tended to be decreased in NASH (Fig. 5, 6).

4 Discussion

ER stress under a severe insult, such as chronic alcohol consumption in hepatocytes, activates a different protein degradation pathways to preserve cell function and viability. Significant upregulation of the first line proteins in ER stress response in NASH compared

to controls and the increasing of the level of the downstream proteins of the ER response in ASH compared to controls, indicates that alcohol toxicity causes more severe cell responses in ASH in comparison to NASH. More gene expression in the downstream UPR pathway in ASH induces autophagocytosis and apoptosis (Fig. 1). In our previous study (Masouminia et al., 2016) we showed an increased level of autophagocytosis and apoptosis in ASH compared to controls and NASH.

MDB formation in hepatocytes is one of the signs of prolonged ER stress and gradual failure of the cytoprotective pathways. Increased expression of BAX as a proapoptotic regulator in the hepatocytes that formed MDBs, indicating an increase in cell death in exhausted hepatocytes.

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Fig. 1.

Cross reaction of cytoprotective pathways and cell death pathways under ER stress

Fig. 2. PERK in ASH and NASH were significantly upregulated ($p < 0.05$) compared to the Controls. The expressions of PERK tend to be elevated in NASH in comparison to ASH. ($\times 690$)

Fig. 3.

The double stain for PERK and UB is shown (A, B). Colocalization of PERK and UB is seen within the Mallory body (C). Also shown is an increase in the fluorescent intensity in the adjacent liver cells. ($\times 520$)

Fig. 4.

IRE-1 α expression in NASH was significantly ($p < 0.05$) elevated compared to controls and tend to be elevated in comparison to ASH. IRE-1 α expressions in ASH tend to be increased compared to the controls. The yellow tracer line converted the fluorescence intensity in to the green line in the graph. ($\times 690$)

Fig. 5.

ATF6 expressions in ASH tend to be increased compared to Controls and NASH. Its expression in NASH was decreased in comparison to the controls. ($\times 690$)

Fig. 6.

The expression of ATF4 in NASH was significantly ($p < 0.05$) downregulated compared to the controls. Its expression in ASH tended to be elevated in comparison to NASH. ($\times 690$)

Fig. 7.

The expression of BAX in ASH with the presence of MDB tended to be elevated in comparison to the controls and ASH without MDB.

Table 1

Antibodies used in the study with animal source and company/Vendor

Antibody	Company/Vendor
<i>PERK</i>	<i>Abcam, Cambridge, MA 02139</i>
<i>IRE-1</i>	<i>Novus Biological, Littleton, CO 80120</i>
<i>ATF4</i>	<i>Abcam, Cambridge, MA 02139</i>
<i>ATF6</i>	<i>Life Span, Seattle, WA 98121</i>
<i>BAX</i>	<i>Abcam, Cambridge, MA 02139</i>