Experimental and Molecular Pathology 98 (2015) 65–72

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Levels of metacaspase1 and chaperones related to protein quality control in alcoholic and nonalcoholic steatohepatitis

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ARTICLE INFO

Article history:
Received 27 November 2014
Accepted 13 December 2014
Available online 16 December 2014

Keywords:
Protein quality control
Alcoholic steatohepatitis
Nonalcoholic steatohepatitis
Mca1
Hsp104
Hsp40
Ydj1
Ssa1
VCP/p97
p62
ERAD
IPOD
JUNQ

ABSTRACT

Efficient management of misfolded or aggregated proteins in ASH and NASH is crucial for continued hepatic viability. Cellular protein quality control systems play an important role in the pathogenesis and progression of ASH and NASH. In a recent study, elevated Mca1 expression counteracted aggregation and accumulation of misfolded proteins and extended the life span of the yeast *Saccharomyces cerevisiae* (Hill et al, 2014). Mca1 may also associate with Ssa1 and Hsp104 in disaggregation and fragmentation of aggregated proteins and their subsequent degradation through the ER-associated degradation (ERAD) pathway. If degradation is not available, protection of the cellular environment from a misfolded protein is accomplished by its sequestration into two distinct inclusion bodies (Kaganovich et al., 2008) called the JUNQ (JUxta Nuclear Quality control compartment) and the IPOD (Insoluble Protein Deposit). Mca1, Hsp104, Hsp40, Ydj1, Ssa1, VCP/p97, and p62 all play important roles in protein quality control systems. This study aims to measure the expression of Mca1 and related chaperones involved in protein quality control in alcoholic steatohepatitis (ASH), and nonalcoholic steatohepatitis (NASH) compared with normal control liver biopsies. Mca1, Hsp104, Hsp40, Ydj1, Ssa1, VCP/p97, and p62 expressions were measured in three to six formalin-fixed paraffin embedded ASH and NASH liver biopsies and control normal liver specimens by immunofluorescence staining and quantified by immunofluorescence intensity. Mca1, Hsp104, Ydj1 and p62 were significantly upregulated compared to control ($p < 0.05$) in ASH specimens. Hsp40 and VCP/p97 were also uptrending in ASH. In NASH, the only significant difference was the increased expression of Hsp104 compared to control ($p<0.05$). Ssa1 levels were uptrending in both ASH and NASH specimens. The upregulation of Mca1, Hsp104, Ydj1 and p62 in ASH may be elicited as a response to the chronic exposure of the hepatocytes to the toxicity of alcohol. Recruitment of Mca1, Hsp104, Ydj1 and p62 may indicate that autophagy, the ERAD, JUNQ, and IPOD systems are active in ASH. Whereas in NASH, elevated Hsp104 and uptrending Ssa1 levels may indicate that autophagy and IPOD may be the only active protein quality control systems involved.

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1. Introduction

Steatohepatitis frequently includes Mallory–Denk Bodies (MDBs), which are an intracellular deposition of misfolded protein in ballooned hepatocytes. Ballooning of hepatocytes is induced by oxidative stress. MDBs are prevalent in various hepatic diseases including hepatitis B and C viral infections, alcoholic steatohepatitis (ASH), non-alcoholic steatohepatitis (NASH), drug injuries and hepatocellular carcinoma (Zatloukal et al., 2007; Basaranoglu, Turhan et al., 2011). Pathological lesions in ASH and NASH are similar. Both can progress to more severe forms of the disease including ballooning of hepatocytes (Caldwell et al., 2010), MDB formation, activation of stellate cells leading to hepatic fibrosis, and ultimately, cirrhosis.

Protein quality control systems play a critical role in the pathogenesis and progression of ASH and NASH. The primary function of protein quality control systems is to detect and efficiently manage misfolded or aggregated proteins in a timely manner for continued cellular function and viability. The process involves recognition of the misfolded protein by chaperones and E3 ligases for ubiquitination and subsequent degradation through various mechanisms. For example, metacaspase 1 (Mca1), with the aid of heat shock protein 104 (Hsp104), counteract the aggregation and accumulation of misfolded proteins (Hill et al., 2014). p62 is involved in linking polyubiquitinated protein aggregates to the autophagy machinery (Bjørkøy et al, 2005). The Hsp70/Hsp40 chaperone system also plays an essential role in cell autophagy. Ydj1 is required for ubiquitin-dependent degradation of certain abnormal proteins. Additionally, Ydj1 interacts with Ssa1 and facilitates ER-associated degradation (ERAD) (Lee et al., 1996). VCP/p97 cooperates with diverse partner proteins to help process ubiquitin-labeled misfolded proteins for recycling or degradation by the 26S proteasome (Bug and Meyer, 2012). However, if such protein degradation mechanisms are unavailable, protection of the cellular environment from a misfolded protein is accomplished by its sequestration into two distinct inclusion bodies (Kaganovich et al., 2008): the JUNQ (JUxta Nuclear Quality control compartment) and the IPOD (Insoluble Protein Deposit).

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http://dx.doi.org/10.1016/j.yexmp.2014.12.003
0014-4800/Published by Elsevier Inc.

ASH and NASH may have different protein quality control systems. This study aims to compare the levels of Mca1, Hsp104, Hsp40, Ydj1, Ssa1, VCP/p97 and p62, all involved in various protein quality control systems. We predicted that ASH and NASH utilize different protein quality control systems.

2. Materials and methods

2.1. Sample selection

Formalin-fixed paraffin embedded (FFPE) human liver biopsy blocks from patients with confirmed ASH and NASH diagnoses were first identified from Harbor–UCLA hospital archives. Normal liver biopsies were also obtained and were used as control. Six ASH, five NASH, and three normal control liver biopsies were used in this study.

2.2. Slide preparation

Tissue slides were then prepared from the FFPE blocks. The slides were double stained for ubiquitin (Millipore, Temecula, CA) and either Mca1, Hsp104, Hsp40, Ydj1, Ssa1, VCP/p97, or p62. Ubiquitin was detected using the red fluorescent (Texas Red) antibody, donkey anti-mouse Alexa Fluor 594 (Jackson Labs, West Grove, PA), while Mca1, Hsp104, Hsp40, Ydj1, Ssa1, VCP/p97, and p62 were detected using the green fluorescent (FITC) antibody, donkey anti-rabbit Alexa Fluor 488 or donkey anti-mouse Alexa Fluor 488 (Jackson Labs, West Grove, PA). The nuclei were stained with DAPI blue. The double stain was detected using a tricolor filter. All biopsies were stained at one time to allow accurate comparisons between groups.

2.3. Microscopy

The fluorescence staining intensity of the proteins were then measured in 3 different areas on each slide. Using a Nikon 400 fluorescent microscope and the Nikon morphometric system, quantitative measurements were taken with 40× objective magnification and a standard exposure time of 800 ms. All photos and measurements were made at the same level of UV light intensity. The results were displayed as a graph attached to the immunofluorescent photography using a screen snip.

2.4. Statistical analysis

All data are presented as mean with S.D. Statistical significance was calculated using one-way ANOVA. Tukey's multiple comparison test was then used to compare significant differences between normal control versus ASH, normal control versus NASH, and ASH versus NASH measurements. A value of $p < 0.05$ denoted statistical significance.

3. Results and discussion

3.1. ASH

Constant and prolonged oxidative stress, lipid peroxidation, and acetaldehyde toxicity from chronic consumption of alcohol result in inflammasome activation and Mallory–Denk Body (MDB) formation (Peng et al, 2014). MDBs are intracellular deposition of misfolded proteins in ballooned hepatocytes, and they occur in 80% of biopsies (French, 1981a, 1981b). All of the ASH liver biopsies showed numerous balloon cells with MBD formation (Fig. 8). In response to oxidative stress and increased lipid peroxidation from chronic alcohol intake, heat shock proteins that identify misfolded or unfolded proteins, and target them for degradation, are expressed. Misfolded and aggregated protein degradation may then proceed via several protein quality control mechanisms, including autophagy and the ERAD, the JUNQ and the IPOD systems (See Fig. 9).

3.1.1. Levels of Mca1, Hsp104, Ydj1 and p62 were significantly elevated in ASH

Compared to normal control biopsies, ASH showed a significant increase in Mca1, Hsp104, Ydj1 and p62 levels (Figs. 1, 2, 4, and 7). Our data suggests that in ASH, the liver may be actively utilizing autophagy and the ERAD, the JUNQ, and the IPOD systems for protein quality control. Mca1, Hsp104, Ydj1 and p62 have different roles in the systems mentioned.

Mca1, Hsp104, Ydj1 and p62 are intra-cellular chaperones. They play an important role in protein–protein interactions such as proper protein conformation and prevention of unwanted protein aggregation. p62, which is also named sequestosome1 (SQSTM1), is a common component of protein aggregates that are found in protein aggregation diseases affecting both the brain and the liver. p62 is involved in the mechanism of MDB formation (Nan et al., 2004). p62 is also involved in linking polyubiquitinated protein aggregates to the autophagy machinery (Bjørkøy et al., 2005) and the protein-degrading complex 26S proteasome (French et al., 2010). Protein inclusions formed by aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by macroautophagy (Kegel et al., 2000; Ravikumar et al., 2002, 2004), which is a bulk degradation pathway in which a double or multimembrane-bound structure called the autophagosome forms to sequester the cytoplasm. Subsequently, the autophagosome fuses with the lysosome, and its content and internal membranes are degraded (Levine and Klionsky, 2004; Yoshimori, 2004). Ydj1 is involved in a variety of cellular activities that control polypeptide fate, such as folding and translocation across intracellular membranes. Ydj1 is also required for ubiquitin-dependent degradation of certain abnormal proteins. Ydj1 is a J-domain containing protein that interacts with Ssa1 and facilitates ER-associated degradation (ERAD). ERAD is a cellular pathway which targets misfolded proteins of the endoplasmic reticulum for ubiquitination and subsequent degradation by the 26S proteasome (Ding and Yin, 2008). Ydj1 may facilitate the recognition of unfolded proteins or may serve as a cofactor for certain ubiquitin-ligating enzymes (Lee et al., 1996). Mca1 may also associate with Ssa1 and Hsp104 in disaggregation and fragmentation of aggregated proteins and their subsequent degradation through ERAD.

Cellular increase of misfolded protein loads, due to the constant presence of oxidative stress and alcohol toxicity, may saturate and exhaust the quality control machinery. For example, it has been shown that chronic intragastric tube feeding of alcohol to rats leads to the inhibition of the 26S proteasome catalytic activity (Bardag-Gorce et al., 2005). In these conditions, a second line of active cellular defense is available. Misfolded proteins may be sequestered into two distinct inclusion bodies: the JUNQ and the IPOD. For example, a previous study showed that when there is low expression levels of the proteasome, ubiquitinated misfolded proteins are sorted into the JUNQ (Kaganovich et al., 2008). Delivery of misfolded and aggregated proteins to JUNQ and IPOD require an intact cytoskeleton and specific cellular quality control components, such as heat shock proteins (Specht et al., 2011). During protoestatic stress, Mca1 is recruited to IPOD and JUNQ (Hill et al., 2014) to sequester terminally unfolded and aggregated proteins. Mca1 may also be recruited with Hsp104, Ssa1, and Ydj1 for JUNQ sequestration of terminally aggregated proteins. Upon recovery from stress conditions, misfolded proteins that accumulated in the JUNQ are either refolded by the cellular chaperone machinery, or degraded by the 26S proteasome. Therefore, sequestration of a misfolded protein to the JUNQ is reversible.

3.1.2. Uptrending of Hsp40 and VCP/p97 in ASH

Although not significantly elevated compared to normal control, Hsp40 and VCP/p97 are significantly elevated in ASH compared to NASH (Figs. 3, 6). Our data suggests that these two chaperones are also involved in the complex protein quality control machinery in ASH, albeit at low levels. Hsp40, also known as Chaperone DnaJ, is a molecular chaperone protein. The J domain of Hsp40 interacts with Hsp70