Experimental and Molecular Pathology 101 (2016) 81–88

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

Upregulation of autophagy components in alcoholic hepatitis and nonalcoholic steatohepatitis

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ARTICLE INFO

Article history:
Received 13 July 2016
Accepted 14 July 2016
Available online 16 July 2016

Keywords:
Mallory-Denk Bodies
Autophagocytosis
Protein quality control
Alcoholic hepatitis

ABSTRACT

There are many homeostatic mechanisms for coping with stress conditions in cells, including autophagy. In many studies autophagy, as an intracellular pathway which degrades misfolded and damaged protein, and Mallory-Denk Body (MDB) formation have been shown to be protective mechanisms against stress such as alcoholic hepatitis. Alcohol has a significant role in alteration of lipid homeostasis, sterol regulatory element-binding proteins (SREBPs) and peroxidase proliferator-activated receptors through AMP-activated protein kinase (AMPK)-dependent mechanism. AMPK is one of the kinases that regulate autophagy through the dephosphorylation of ATG1. Activation of ATG1 (ULK kinases family) activates ATG6. These two activated proteins relocate to the site of initial autophagosome and activate the other downstream components of autophagocytosis. Many other proteins regulate autophagocytosis at the gene level. CHOP (C/EBP homologous protein) is one of the most important parts of stress-inducible transcription that encodes a ubiquitous transcription factor. In this report we measure the upregulation of the gene that are involved in autophagocytosis in liver biopsies of alcoholic hepatitis and NASH. Electron microscopy was used to document the presence of autophagosomes in the liver cells. Expression of AMPK1, ATG1, ATG6 and CHOP in ASH were significantly (p value < 0.05) upregulated in comparison to control. Electron microscopy findings of ASH confirmed the presence of autophagosomes, one of which contained a MDB, heretofore undescribed. Significant upregulations of AMPK-1, ATG-1, ATG-6, and CHOP, and uptrending of ATG-4, ATG-5, ATG-9, ATR, and ATM in ASH compared to normal control livers indicate active autophagocytosis in alcoholic hepatitis.

1. Introduction

Autophagy is one of the essential intracellular pathways that maintains cellular functions and prevents cell death via lysosomal degradation as a cytoprotective path. More than 30 ATG genes are identified for controlling autophagocytosis (Klionsky et al., 2003). ATG1–10 are participating as core proteins in autophagosome formation (Nakatogawa et al., 2009) that have four subgroups: a) ATG1/ULK1 complex (Chan, 2012) that induces autophagocytosis (Russell et al., 2013) and is negatively modulated by mTOR; b) ATG6/Beclin1 complex (Di Bartolomeo et al., 2010) that regulates the formation of autophagosome; c) Ubiquitin-like complexes (LC3 and ATG12-5) regulate vesicle expansion; d) ATG9 that is required for delivery of membranes which form autolysosome (Xie and Klionsky, 2007; Fujita et al., 2008; Hosokawa et al., 2009; Yang and Klionsky, 2009; Yang and Klionsky, 2010). Autophagy plays a very important role in hepatocytes homeostasis by removing misfolded proteins and damaged organelles. Accumulation of proteins in prolonged ER stress may lead to intra hepatic protein aggregate formation such as MDBs (Komatsu, 2012; Liu et al. 2014; Peng et al. 2014). ATP and energy level reduction in the cell, increase AMPK related autophagy by removing the inhibitory brakes on mTORC1 or activating ULK1 directly (Mihaylova and Shaw, 2011). mTOR has some regulatory effects on liposynthesis; its suppression increases lipophagy and decreases lipogenesis via regulation of SREBP (Peterson et al., 2011). Excess fat deposition, compromised lipid metabolism in NASH and chronic alcohol consumption which plays a significant role in alteration of lipid homeostasis, SREBP and peroxidase proliferator-activated receptors in ASH activate cell stress responses through the AMPK-dependent mechanism (Ceni et al., 2014). AMP binds to the activated part of AMPK when cell energy is low; phosphorylation of AMPK inactivates mTORC1 and activates ULK1 family (ATG1) (Kim et al., 2011). ATG1/ULK1 complex with the support of other proteins translocates to the autophagosome formation sites (Hara et al., 2008), regulates Beclin1/ATG6 and translocates ATG9 from Golgi as an additional membrane donor for autophagosome formation (Young et al., 2006). ATG4 and ATG5 are other ATG proteins interact with the LC3 complex in autophagocytosis (Zhang et al., 2016). Peroxisomes are organelles necessary for β-oxidation of fatty acids, which produces lots of reactive oxygen species (ROS), peroxisomes

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http://dx.doi.org/10.1016/j.yexmp.2016.07.002
0014-4800/

Table 1
Antibodies used in the study with animal source and company/vendor.

Antibody	Company/vendor
AMPK1	SigmaAldrich, St. Louis, MO 63103
ATG1	Thermo-Fisher Rockford, IL 61105
ATG4	MyBioSource, San Diego, CA 92195
ATG5	NovusBio, Littleton, CO 80120
ATG6	SigmaAldrich, St. Louis, MO 63103
ATG9	Abcam, Cambridge, MA, 02139
ATR	LSBio, Seattle, WA 98121
ATM	Abcam, Cambridge, MA 02139
CHOP	Thermo-Fisher, Rockford, IL 61105

usually degrade via autophagy. ATM serine/threonine kinase (ATM) is the first responder that is activated by peroxisomal ROS. One of the actions of activated ATM is signaling AMPK and subsequently activates ATG1 (Tripathi et al., 2016). Any kind of stress and cell insult may lead to DNA damage. The cell response to DNA damage activates the DNA damage checkpoint, which induces cascades of proteins and autophagocytosis, controlled by Mec1 kinase (ATR) (Eapen et al., 2015). CHOP expression is upregulated during cell and ER stress. CHOP has been shown to be involved in the cell death via apoptosis pathway. However, CHOP also has been determined as a regulator in autophagy (Zinszner et al., 1998; Li et al., 2014; Zheng et al., 2014; Ohoka et al., 2005; Su and Kilberg, 2008) CHOP can modify DNA binding activities in the nucleus and regulate transcriptional factor involving to autophagocytosis or apoptosis (Bruhat et al., 1997). Longevity of stress may increase the expression of cell death mediators as autophagy mediators in the long term stress activate apoptosis regulators (Fimia and Piacentini, 2010). The aim of this study is to quantitate the expression of the proteins participating in autophagocytosis in alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) in comparison to normal liver.

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 {"Alcoholic hepatitis pathogenesis as determined from human liver tissue analysis" exempted as determined by the iRIS system} were compared to controls. In this study we used 8–12 ASH, 1–5 NASH and 3 normal liver controls. The slides were double stained for ubiquitin plus AMPK1, ATG1, ATG4, ATG5, ATG6, ATG9, ATM, ATR and CHOP. 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 (Jackson Labs, West Grove, PA). The nuclei were stained by DAPI. The staining was done at the same time for all slides together to provide accurate comparisons between groups. We measured the intensity of the fluorescent staining in 3 different areas on each slide with 40× magnifications and 800 ms standard exposure time by using a Nikon 400 fluorescent microscope. The Nikon morphometric system was used to a quantitate measurement of the fluorescent intensity morphometrically. The mean, standard error and statistical differences of data achieved from the Nokia were analyzed by Graph pad statistical software. Controls vs. ASH, controls vs NASH and ASH vs. NASH were compared by unpaired *t*-test with $p < 0.05$. Electron microscopy was used to document the presence of autophagosomes in the liver cells (see Table 1).

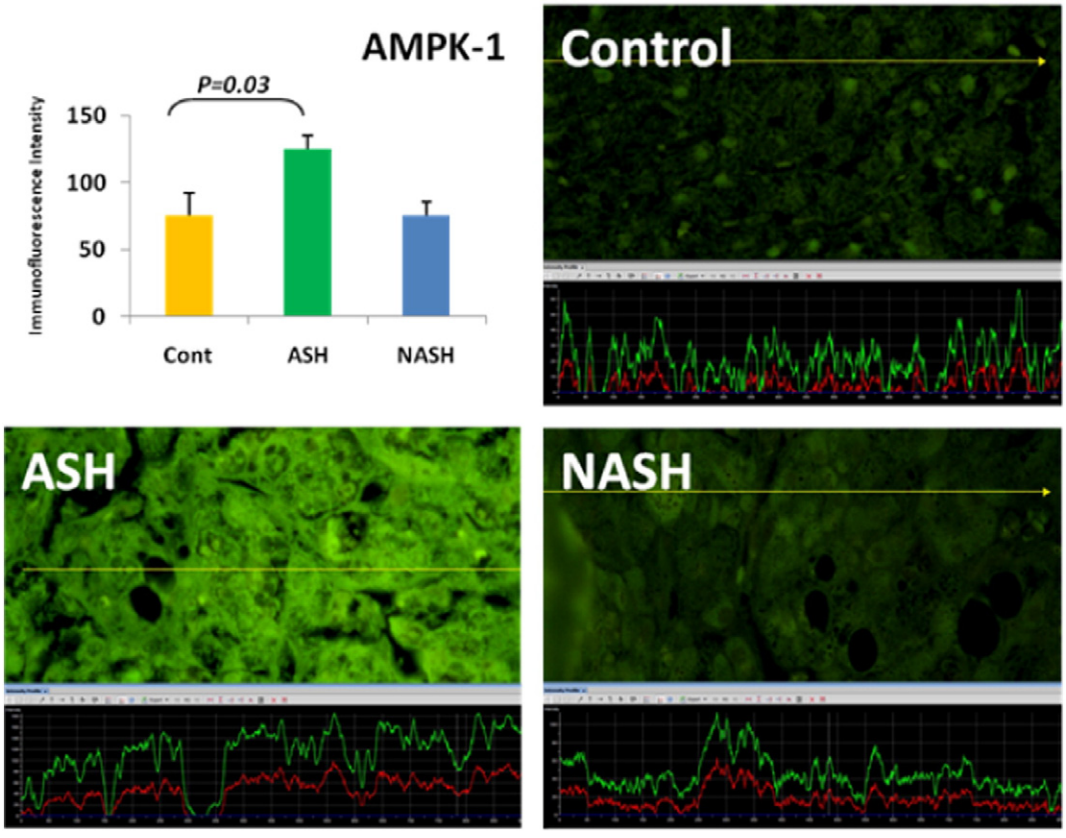

Fig. 1. AMPK-1 was significantly upregulated compared to controls ($p < 0.05$). The yellow tracer line converted the fluorescence intensity in to the green line in a graph (×690).