Annals of Hepatology 28 (2023) 100900

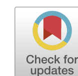
Contents lists available at ScienceDirect

Annals of Hepatology

journal homepage: www.elsevier.es/annalsofhepatology

Opinions

Targeting cellular senescence as a therapeutic approach in non-alcoholic steatohepatitis



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Chronic liver disease is the 11th leading cause of death and affects 2 million people annually worldwide. The causes of chronic liver injury include hepatitis B virus (HBV), hepatitis C virus (HCV), nonalcoholic fatty liver disease (NAFLD), and alcoholic liver disease [1]. In recent years, the number of patients with viral hepatitis has been declining owing to the development of highly effective antiviral therapies against HBV/HCV and the widespread use of HBV vaccines. On the other hand, the prevalence of NAFLD, such as non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), is increasing due to the global epidemic of obesity and type 2 diabetes mellitus [2]. NAFL does not show particular pathological symptoms in its simple fatty deposit stage and has a good prognosis. However, when NAFL is combined with several other stressors, NASH develops, which causes persistent inflammation of the liver and progresses to cirrhosis and cancer [3]. As no effective treatment is currently available for NASH, elucidating the mechanisms that regulate NASH pathogenesis is an important research issue.

Cellular senescence is a stable cell cycle arrest caused by a variety of stimuli including persistent telomere shortening (replicative senescence), activation of oncogenes, oxidative damage, and DNA damage by radiation and chemotherapy [4]. Other notable features of senescent cells include apoptosis resistance, mitochondrial dysfunction, and senescence-associated secretory phenotype (SASP) [5]. SASP produces a variety of secreted proteins (inflammatory cytokines, chemokines, extracellular matrix degrading enzymes, other proteases, growth factors, etc.) [6]. SASP factors released from senescent cells have been suggested to induce liver inflammation and exacerbate the pathogenesis of NASH. These senescence-specific phenotypes have been characterized primarily using *in vitro* culture systems; however, *in vivo* characterization has been difficult due to the lack of appropriate tools to identify and isolate senescent cells from the liver.

Senescent cells express $p16^{Ink4a}$, a protein that inhibits cyclin-dependent kinases and arrests the cell cycle. Previous reports have shown selective elimination of $p16^{Ink4a}$- positive cells using transgenic approaches or pharmacological methods, such as senolysis, prolonged healthy life span and amelioration of various aging-related diseases [7]. These results indicate that accumulation of senescent cells promotes the onset and worsening of age-related diseases. To characterize p16-expressing senescent cells *in vivo*, we generated knock-in mice with $CreER^{T2}$ at the $p16^{Ink4a}$ gene locus [8]. The p16-$CreER^{T2}$-tdTomato mouse, generated by crossing the p16-$CreER^{T2}$ mouse with the Rosa26-LSL-tdTomato mouse, can tag p16-expressing senescent cells with the red fluorescent protein, tdTomato, in a tamoxifen-dependent manner. NASH was induced in these mice using a choline- deficient amino acid-defined high-fat diet (CDA-HFD). Senescent non-parenchymal cells were sorted from the liver by tdTomato expression, and single-cell gene expression was performed using single-cell RNA-sequencing (scRNA-seq). The results showed that tdTomato-positive cells were distributed among cell types, including sinusoidal endothelial cells, Kupffer cells, macrophages, and hepatic stellate cells, in normal and NASH livers. Interestingly, some senescent sinusoidal endothelial cells were found to combine the characteristics of Kupffer cells. In NASH, tdTomato-positive cells had significantly higher numbers of macrophages than those in normal livers. GO analysis revealed the enrichment of molecules related to interleukin-6 (IL-6) production and regulation of NF-κB in the upregulated genes in tdTomato-positive macrophages. These results are consistent with SASP characteristics and indicate that the pathological progression of NASH leads to the accumulation of senescent macrophages. Therefore, we generated p16-CreERT2-DTR mice by crossing mice that expressed Cre-dependent human diphtheria toxin receptor (Rosa26-LSL-DTR) with p16-CreERT2 mice. NASH was induced in these mice using CDA-HFD and then treated with tamoxifen and diphtheria toxin. As a result of this senolysis, the lipid droplet area in hepatocytes and the number of macrophages markedly decreased. These results suggested that senolysis may be an effective treatment for NASH. Next, we investigated the possibility of treating NASH with drug-induced senolysis.

Since the first report of senolysis with co-administration of dasatinib and quercetin, several senolytic drugs have been identified [9]. We also found that glutaminase-1 (GLS1) is specifically activated in senescent cells and confirmed that administration of the GLS1 inhibitor BPTES eliminates senescent cells *in vivo* and ameliorates the symptoms of aging-related diseases, including NASH [10]. Although the Bcl-2 family inhibitors ABT-737 and

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https://doi.org/10.1016/j.aohep.2023.100900
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ABT-263 are effective in eliminating senescent cells in animals, Bcl-XL inhibition is known to cause severe pancytopenia [11]. Therefore, the clinical application of senolytic drugs should be carefully considered. Since hepatic function is impaired in NASH, drugs may not be metabolized properly.

We investigated the mechanism by which senescent cells are not eliminated from the body using senescent cells from p16-CreER^{T2}-tdTomato mice. We found that some senescent cells expressed programmed cell death ligand 1 (PD-L1), an immune checkpoint protein [12]. The ratio of PD-L1-expressing senescent cells significantly increased with age, suggesting that the PD-L1-mediated immune escape mechanism is involved in the accumulation of senescent cells. Immune checkpoint blockade (ICB) therapy using monoclonal antibodies against PD-1 and PD-L1 has contributed to major advances in cancer immunotherapy over the past decade. We administered anti-PD-1 antibodies to aging mice and successfully eliminated senescent cells *in vivo*. Next, the effect of the anti-PD-1 antibody on NASH induced using CDA-HFD was examined, and a more remarkable improvement in NASH pathology was observed compared to that of ABT-263.

These results clearly indicated that the elimination of senescent cells is a target for the treatment of NASH. On the other hand, analysis using p16-CreER^{T2}-tdTomato mice revealed that senescent cells accumulating with age include many non-proliferative cell types, such as neurons and cardiomyocytes, which are difficult to regenerate (personal communication with lab members). This suggests that senolysis is not always an optimal therapeutic strategy. Recently, SASP and other characteristics of senescent cells have been reported to be associated with the progression of NASH [13,14]. Identification of senescent cell-derived aggravating factors, rather than the elimination of senescent cells, will be the target of future NASH therapeutic strategies.

Declaration of interest

None

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