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Liver Regeneration as Treatment Target for Severe Alcoholic Hepatitis

Liver Regeneration for Treatment of Alcoholic Hepatitis

Abstract

Severe alcoholic hepatitis (AH) is a distinct entity in the spectrum of alcohol-related liver disease, with limited treatment options and high mortality. Supportive medical care with corticosteroids in selected patients is the only currently available treatment option, often with poor outcomes. Based on the insights into the pathogenetic mechanisms of AH, which are mostly obtained from animal studies, several new treatment options are being explored. Studies have implicated impaired and deranged liver regeneration processes as one of the culprit mechanisms and a potential therapeutic target. Acknowledging evidence for the beneficial effects of granulocyte colony-stimulating factor (G-CSF) on liver regeneration and immunomodulation in animal models, several human studies investigated its role in the treatment of advanced alcohol-related liver disease and AH. Contrary to the previously published studies suggesting benefits of G-CSF in the outcomes of patients with severe AH, these effects were not confirmed by a recently published multicenter randomized trial, suggesting that other options should rather be pursued. Stem cell transplantation represents another option for improving liver regeneration, but evidence for its efficacy in patients with severe AH and advanced alcohol-related liver disease is still very scarce and unconvincing, with established lack of efficacy in patients with compensated cirrhosis. In this review, we summarize the current knowledge on the pathogenesis and experimental therapies targeting liver regeneration. The lack of high-quality studies and evidence is a major obstacle in further treatment development. New insights into the

pathogenesis of not only liver injury, but also liver regeneration processes are mandatory for the development of new treatment options. A reliable experimental model of the pathogenesis of AH and processes involved in liver recovery is still missing, and data obtained from animal studies are essential for future research.

Key Words: Severe alcoholic hepatitis; Alcohol-related liver disease (ALD); Treatment; Liver regeneration; Granulocyte colony-stimulating factor (G-CSF); Stem cell transplantation

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Core Tip: Current treatment options for patients with severe alcoholic hepatitis (AH) are unsatisfactory, resulting in high mortality rates. Liver regeneration is impaired in patients with severe AH and represents an appealing therapeutic target. Granulocyte-colony stimulating factor (G-CSF), alone or in combination with stem cell therapy represents a promising therapeutic option for severe AH. Contrary to animal and several small human studies, evidence from recent studies failed to show benefit of these liver regeneration therapies in patients with advanced alcohol related liver disease.

INTRODUCTION

The harmful use of alcohol is responsible for more than 3 million deaths every year (5.3% of all-cause mortality) and is one of the most common causes of liver disease worldwide[1,2]. Excessive alcohol consumption can cause a spectrum of partially overlapping liver injuries, including steatosis, steatohepatitis, fibrosis and cirrhosis. All these histological changes with accompanying clinical manifestations are collectively termed alcohol-related liver disease (ALD).

Alcoholic hepatitis (AH) is a distinct clinical manifestation of ALD, characterized by jaundice and sometimes accompanied with other signs of hepatic decompensation and liver failure such as ascites or encephalopathy, which occurs in patients with excessive alcohol consumption[3]. Underlying this clinical condition are histological changes including steatosis, hepatocyte injury with ballooning degeneration and lobular neutrophil infiltration, which are characteristic of alcoholic steatohepatitis, and which usually occur in the presence of advanced fibrosis or cirrhosis[3]. The prognosis of AH is generally dismal but significantly dependent on the severity of the disease. Patients with severe forms of AH (defined by Maddrey's Discriminant Function ≥32) have a significantly worse prognosis with mortality rates reported between 20% and 30% after one month, and 30% to 40% after six months from presentation[4]. Although patients with a non-severe form of AH have a <10% risk of mortality after one month and survival of patients with severe AH seems to have improved over the last decades, the condition still carries a poor prognosis[3].

CURRENT TREATMENT OPTIONS FOR SEVERE ALCOHOLIC HEPATITIS

Patient stratification based on the disease severity and prognosis is necessary for treatment decisions in AH. A Maddrey's Discriminant Function ≥32, Model for End-Stage Liver Disease (MELD) score ≥21, and/or Glasgow Alcoholic Hepatitis (GAH) score ≥9 are models most commonly used to identify patients with severe disease.

Alcohol abstinence and appropriate nutrition are the cornerstone of treatment of AH, regardless of disease severity[3,5,6]. Energy intake of 30-40 kcal/kg/day and protein intake of 1.2-1.5 g/kg/day are currently recommended by most treatment guidelines. Treatment of alcohol withdrawal symptoms, hepatic encephalopathy, ascites and other comorbidities with vitamin B supplementation also represent general measures required in many patients.

For many years, corticosteroids have been used in the treatment of patients with severe forms of AH, with varying results. A large, multicenter randomized trial comparing steroids with pentoxifylline (STOPAH) showed modest reduction in

mortality after 28 days of treatment with prednisolone compared to pentoxifylline, but no improvement in long-term outcomes was observed after 90 days and 1 year[7]. These findings were also confirmed by a recent meta-analysis[8]. Wide use of corticosteroids is limited not only by scarcity of data on its efficacy, but also by safety concerns, especially regarding infections and risk of gastrointestinal bleeding. However, corticosteroids are considered the only treatment option for severe AH with proven or likely benefit[3,5,6].

Patients with severe AH who fail to respond to standard supportive care and corticosteroids may require liver transplantation. However, since the diagnosis of AH implies recent alcohol abuse, these patients are not readily accepted for liver transplantation by most transplant centers because of inadequate abstinence period. Nonetheless, an increasing number of centers are considering and performing liver transplantation in highly selected patients with severe AH[9,10]. The survival benefit for transplanted patients with severe AH has been demonstrated in several trials and a model study, and concern of alcohol abuse relapse after early transplantation has been addressed in several small studies, which showed similar relapse rates to those recorded in patients with 6-month abstinence period in highly selected groups of patients[11,12]. Still, liver transplantation is not an option for the majority of patients with severe AH.

PATHOPHYSIOLOGY OF LIVER INJURY AND LIVER REGENERATION

The spectrum of alcohol related liver damage results from an interplay of several interrelated pathophysiological mechanisms caused by continuous excessive alcohol consumption. Direct injury to hepatocytes and inflammation cause a series of consecutive events resulting in histological changes characteristic for ALD[13,14]. Ethanol metabolites, namely acetaldehyde, can cause direct damage to hepatocytes through oxidative stress, lipid peroxidation, mitochondrial dysfunction and alterations in lipid metabolism, resulting in steatosis. Consequently, damaged hepatocytes activate apoptosis and necrosis pathways, which result in the release of Damage-Associated Molecular Patterns (DAMPs), cell derived molecules that trigger the immune system,

which causes further damage. Another mechanism involved in the pathogenesis of ALD results from a deranged gut-liver axis[15]. Increased gut permeability and dysbiosis caused by alcohol consumption allow for translocation of lipopolysaccharides and other bacterial products, collectively termed Pathogen-Associated Molecular Patterns (PAMPs), through splanchnic vasculature into the liver. Stimulation of resident macrophages in the liver (Kupffer cells) by PAMPs and DAMPs through activation of toll-like receptors (TLRs) enhances nuclear factor kappa-B (NFkB) signaling and results in expression of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6[16]. These proinflammatory cytokines together with chemokines released by Kupffer and other liver cells recruit neutrophils and other immune cells that infiltrate liver parenchyma and contribute to secondary liver injury. Chemokine receptors also promote inflammation through NFkB signaling. All these triggers that activate Kupffer cells and other inflammatory cells also stimulate the release of platelet-derived growth factor and transforming growth factor-β (TGF-β). These profibrotic cytokines stimulate hepatic stellate cells and myofibroblasts to produce collagens, thus promoting fibrosis with consequent distortion in the liver architecture, which is a hallmark of cirrhosis. Activated immune cells induce hepatic injury by formation of reactive oxygen species, which are responsible for oxidative stress. Ethanol metabolism also changes the redox state of hepatocytes and increases their vulnerability to free radicals by depletion of antioxidant storage.

At the same time when hepatic injury causes hepatocyte apoptosis and necrosis, it also stimulates liver regeneration. The liver is an organ with unique regenerative capacity and employs multiple mechanisms for regeneration in order to restore functional tissue following insults such as toxic injury or partial hepatectomy[17]. The knowledge on the pathophysiology of liver damage in AH and insights into the liver regeneration processes mostly come from animal studies[17,18]. However, the existing animal models do not reproduce the main histological features of poor prognosis in patients with severe AH, such as severe fibrosis and bilirubinostasis, which are necessary to understand the processes occurring in such a milieu[19].

It is presumed that damaged hepatocytes release signals which trigger liver regeneration processes in order to restore functional liver parenchyma[20]. Multiple extracellular signals regulating hepatocyte proliferation during liver regeneration have been identified[17]. Among them, hepatocyte growth factor (HGF) and its receptor, ligands of the epidermal growth factor (EGF) receptor and transforming growth factor-α (TGF-α) seem to have a major role. Other signaling pathways include tumor necrosis factor-α (TNF-α) and its receptor, IL-6, vascular endothelial growth factor (VEGF) and its receptors, several complex signaling pathways such as Hedgehog pathway, Wnt and β-catenin, Hippo and Yap pathway, as well as several other hormones and signaling molecules[17]. In their absence, the regeneration process will be impaired and delayed but not completely inhibited. In animal models, the first intracellular signaling pathways activated following partial hepatectomy include β-catenin and Notch pathways, followed by activation of signal transducer and activator of transcription 3 (STAT3) and NFκB, which are regulated by IL-6 and TNF-α[17]. Although different mechanisms may activate these pathways, the exact triggers have not been clearly established.

It seems that local, paracrine signals between proliferating hepatocytes and other cell types such as hepatic stellate cells and liver sinusoidal endothelial cells are also important for regeneration. Thus, hepatocytes are at the center of orchestrated liver regeneration processes aimed to restore histologically complete liver tissue, and all liver cell types are able to proliferate during the process. VEGF and angiopoietins are mitogenic for liver sinusoidal endothelial cells, TGFα for sinusoidal endothelial and hepatic stellate cells, fibroblast growth factor 1 and 2 for stellate cells and sinusoidal endothelial cells, and granulocyte-macrophage colony-stimulating factor (GM-CSF) for Kupffer cells. For sinusoidal endothelial and Kupffer cells, there is evidence that precursor cells migrating from the bone marrow may also be involved in the regeneration process, in addition to local cell proliferation[17].

Normal hepatocyte proliferation and liver regeneration processes, however, seem to be less effective in ALD. Chronic ethanol exposure impairs liver regeneration

by interfering with normal microRNA signaling and DNA synthesis, thus inhibiting mature hepatocyte proliferation[14].

The lack of a reliable experimental model with abnormal liver regeneration makes it difficult to understand how these processes affect alcohol-injured livers. It has been proposed that in severe injury, hepatocytes might be unable to initiate the regeneration and tissue healing by themselves, which is then stimulated by liver progenitor or oval cells that become activated and give rise to hepatocytes and other non-parenchymal liver cells[21]. Furthermore, some data suggest that under conditions where normal regeneration processes are impaired by alcohol exposure, liver progenitor cells may accumulate in damaged livers, but are unable to differentiate into mature hepatocytes, which might explain why some patients with severe AH fail to improve. This hypothesis has been corroborated by the results of a human study on tissue samples obtained from explanted livers of patients with severe AH having undergone salvage liver transplantation[22]. The authors showed that patients with severe AH who were unresponsive to medical treatment had decreased expression of TNF-α and IL-6, the cytokines shown to be involved in liver regeneration, as well as an aberrant regeneration process in which accumulated hepatic progenitor cells showed predominantly cholangiocyte differentiation and failed to produce mature hepatocytes[22].

All these pathophysiological mechanisms responsible for liver damage and ineffective regeneration associated with ALD are the potential treatment targets in AH. A number of molecules and treatments have been investigated in the attempts to stop and reverse deleterious effects of alcohol in the development and progression of changes associated with ALD[23].

EXPERIMENTAL OPTIONS TARGETING LIVER REGENERATION FOR THE TREATMENT OF SEVERE ALCOHOLIC HEPATITIS

Liver regeneration represents one of the key targets for the development of new treatments for severe AH. Hepatocyte damage and death in AH should be

counterbalanced by liver regeneration, in order to restore normal liver structure and function. However, ineffective liver regeneration has been proposed as the culprit for progressive liver failure in patients with severe AH. Recognizing complex mechanisms involved in liver regeneration processes, a number of treatment targets are currently being explored and developed.

Granulocyte colony-stimulating factor (G-CSF)

Granulocyte colony-stimulating factor (G-CSF) stimulates bone marrow to produce neutrophils and stem cells (SCs), which are consequently released into the blood. In animal models of toxic liver injury, G-CSF administration was shown to mobilize hematopoietic SCs, ameliorate liver injury, promote liver regeneration, and improve liver function and survival.

In a murine model of acute and chronic liver injury, G-CSF accelerated liver regeneration predominantly by promoting proliferation of endogenous hepatocytes, and the process was to a smaller degree mediated by bone marrow originating SCs[24]. In a recently published study in mice exposed to alcohol and irradiation, pretreatment with G-CSF was associated with enhanced macrophage infiltration, preserved tissue structure and function, and improved survival. G-CSF pretreatment stimulated liver regeneration, as shown by immunofluorescence staining for liver SCs. Interestingly, higher expression of hepatic progenitor cells was observed in the injured but untreated control group. This could confirm previous findings that progenitor cells accumulate in response to injury, but are unable to restore normal tissue and function without stimulation[25]. Moreover, G-CSF treatment in mice may mitigate radiation induced fibrosis[26]. Although not based on disease models fully consistent with AH, these animal studies underpin G-CSF as a potential therapeutic strategy in the treatment of liver disease.

In human studies, G-CSF was used as an induction agent for SC recruitment, or as therapy for liver failure by itself, for its other beneficial effects not attributable to SC mobilization, such as improved liver regeneration and neutrophil function. The most

important human studies using G-CSF in order to improve liver regeneration and outcomes in ALD are summarized in Table 1.

In a small randomized trial in patients with alcoholic cirrhosis and biopsy-proven AH, G-CSF showed increased CD34+ stem cell mobilization, increased hepatocyte-growth factor (HGF), and proliferation of hepatic progenitor cells compared to control group, but liver histology, neutrophils and levels of proinflammatory cytokines (IL-6, TNF-α) remained similar[27].

In a randomized controlled trial in patients with acute-on-chronic liver failure (ACLF), G-CSF increased the numbers of CD34+ cells in the livers of treated patients, improved clinical outcomes including reduced Child-Turcotte-Pugh (CTP) and Model for End-stage Liver Disease (MELD) scores, and showed lower rates of hepatorenal syndrome, hepatic encephalopathy or sepsis in treated patients compared to control group[28]. Similar effects were confirmed in a randomized open-label study from India in patients with severe AH, where treatment with G-CSF showed increased counts of CD34+ cells in peripheral blood and ameliorated both CTP and MELD scores, as well as Maddrey's Discriminant Function values after 1, 2 and 3 mo. More importantly, G-CSF therapy increased the 90-day survival rate of patients with severe AH, compared to standard treatment[29]. Five-day dosing regimen of G-CSF has been most widely used, but multiple cycle G-CSF therapy seems appealing, as it may extend the bridging period to transplantation. Only one trial assessed the effects of multiple cycle therapy compared to standard medical therapy in patients with decompensated alcoholic cirrhosis. Repeated treatment cycles were not associated with serious adverse events, but significantly reduced the risk of infections, improved ascites control, and had a positive effect on the patients' quality of life[30].

Consequent studies explored the possible synergistic effects of G-CSF in combination with other immunostimulants such as erythropoietin or plerixafor. In mice, treatment with G-CSF, plerixafor or a combination of G-CSF with plerixafor showed increased circulating hematopoietic SCs with various liver engrafting potential for tested agents, but reduced degree of fibrosis in all treatment groups. However, bone

marrow derived hepatocytes and SCs were detected with low frequencies, suggesting alternative mechanisms in liver regeneration[31]. Results of a study investigating combination therapy with G-CSF and plerixafor in patients with end-stage liver disease are unknown[32].

In a randomized controlled trial in patients with decompensated cirrhosis treated with G-CSF and erythropoietin compared to G-CSF alone, combination therapy showed significant improvements in CTP and MELD scores, as well as a reduced incidence of renal injury, encephalopathy and ascites episodes. In addition, combination therapy altered both cellular and protein expression patterns in liver tissue, leading to higher Ki67+ proliferation index, macrophage infiltration rates, and decreased markers of fibrosis progression[33]. However, some clinical trials, as well as animal studies, suggest that these improvements are not solely related to effective cell proliferation.

Apart from its direct effects on liver regeneration, G-CSF is a readily used agent for stimulating SC mobilization and grafting. The mechanisms affecting SC mobilization are thought to be multifaceted and dependent on the interplay between changes in the liver tissue and bone marrow settings. In bone marrow, SC receptors and anchoring mediators are responsible for keeping them nested and quiet. Since cell mobilization can be sufficient in mice without G-CSF receptors expressed on their hematopoietic SCs, G-CSF impact might be achieved indirectly by altering the stromal bone marrow environment as well[34]. End-stage liver disease might undermine mobilization effects of G-CSF due to spleen enlargement and bone marrow dysfunction as a concomitant feature of end-stage liver disease. A case-control study in patients with AH showed weaker response in CD34+ cell recruitment in comparison to healthy controls[27]. Examination of bone marrow specimens of patients with liver cirrhosis revealed altered SC function, which might be the cause of lower mobilization rates, especially when combined with increased levels of proinflammatory cytokines[35].

Stem cell therapy

Stem cells and tissue engineering have become an attractive field of medical research. Current insights into molecular aspects of hepatic injury of various etiologies bolster SCs as a novel and promising strategy for the treatment and restoration of damaged liver. Traits of liver progenitor cells, often called oval cells, were described in animal toxicity studies investigating hepatocyte reaction in the course of carbon tetrachloride, galactosamine and N-nitrosomorpholine exposure[40]. Increase in α-fetoprotein (AFP) and thymidine tracing of immature hepatocytes suggested that regenerated cells were derived from oval cells, which have a pivotal role in the rat liver tissue response to injury[41]. In humans, those cells correspond to intermediate hepatobiliary cells presumably residing in canals of Hering, and were proven to have capacity for differentiating into both hepatocytes and bile duct cells. They are frequently found in structures called ductular reactions in cirrhotic liver[42,43]. They are usually called liver progenitor cells or intrinsic progenitor cells to distinguish them from non-hepatic SCs derived from bone marrow or other tissues. Human liver progenitor cells exhibit markers during immunophenotyping, which are different from those in oval cells in rats, while simultaneously failing to express AFP, a marker commonly used in animal studies to assess the hepatocyte repair rate. AFP is classified as an oncofetal protein in humans and is produced during embryonic development by the liver and the yolk sac, from which hematopoietic cell lineage derives[44]. Moreover, partial hepatectomy and exposure to carcinogens enhance SC factor production by oval cells, indicating genetic similarity between them and hematopoietic SCs[45]. Although some authors, based on animal studies, dispute the role of hepatic progenitor cells in liver regeneration, most studies suggest that they become important in severe liver injury, as occurs in AH[17]. Indeed, it has been demonstrated that the origin of hepatocytes during liver regeneration varies in different models, and that although hepatocytes are the main drivers for regeneration under normal conditions, an alternative regeneration process may be initiated by liver progenitor cells[46].

Using SCs for liver regeneration has several advantages; they are easily obtained from the patient, can be cryopreserved, self-renewed and are plentiful, and therefore,

they represent a safe and inexpensive treatment option that can be easily repeated and is not related to ethical concerns frequently seen in orthotopic liver transplantation (LT). Cell-based therapy relies mostly on stem or progenitor cells because mature hepatocytes are scarce and are unable to engraft successfully[47]. Although hepatocyte transplantations have been performed, mostly using cell grafts isolated from livers unsuitable for LT, and despite many advantages, the shortage of high-quality grafts, histological incompatibility, the need for immunosuppression and unproven long-term benefits are major drawbacks that prevent their clinical use and impose search for other alternatives.

In humans, stem and progenitor cells involved in the liver regeneration process are classified as hepatic and non-hepatic. Liver progenitor cells, as previously mentioned, correspond to oval cells described in rats. Non-hepatic progenitor cells represent a wide range of cells arising from different tissues. Mesenchymal SCs and hematopoietic SCs found in bone marrow represent the most frequently used SCs[46].

Animal and *in vitro* studies are the cornerstone for investigating beneficial effects of SCs in liver injury. The proposed mechanisms for alleviating liver injury include hepatocyte growth stimulation and cell differentiation, paracrine impact on neighboring cells, reduction in inflammatory cytokine production, and counteracting fibrosis by metalloproteinase expression[48,49]. Cell stemness has been noted in several cell types, *e.g.*, hepatoblasts, liver progenitor cells, hematopoietic and mesenchymal SCs. *In vitro* expanding and harvesting of hepatoblasts in mice, as a counterpart to human liver progenitor cells, is not promising[50]. On the other hand, induced pluripotent SCs were able to produce hepatocytes similar both structurally and functionally to the ones seen in wild type mice[51].

Sepsis-related injury in mice can be reduced by using bone marrow derived mesenchymal SCs, which restore liver function and architecture and enhance immunosuppressive cytokine production, thus increasing the levels of transcription factors important in the oxidative stress cascade[52]. In animal studies, endothelial progenitor cells in combination with hematopoietic SCs have also been used to reduce

sinusoidal endothelial cell injury, which was not described in human studies[53]. In mice, amelioration of small-for-size graft injury was achieved by adipocyte derived SCs owing to their ability to enhance VEGF production[54].

In the fibrosis model caused by carbon-tetrachloride, mesenchymal SC application led to decrease in apoptosis, fibrosis and immune cell tissue infiltration, especially macrophages, which was also proven in animal studies of AH[55, 56].

Current trials in humans report using therapeutic strategies based on mesenchymal SCs (mostly from bone marrow and adipose tissue) and hematopoietic SCs. The summary and key findings of clinical studies using SCs in the treatment of ALD are summarized in Table 2.

Hematopoietic stem cells

Hematopoietic SCs were the first one used in liver tissue engineering owing to their ability of homing in liver tissue. Efficient *in vitro* differentiation of hematopoietic SCs to hepatocytes in a period of several days supported this theory[74]. Engrafted in the livers of mice, hematopoietic SCs demonstrated great ability to repopulate, indicating that hematopoietic SCs may be used as cell reconstruction therapy beyond the scope of hematologic malignancy treatment[75]. After anchoring in the liver tissue, hematopoietic SCs showed a potential to transdifferentiate to hepatocytes, as well as to fuse with tissue host cells, thus changing the cell genetic properties[76]. Hepatocyte growth factor (HGF) is an important paracrine regulator of cell growth, embryologic development, as well as cell regeneration process. In hepatocytes, it renders antiapoptotic effects. Correlations between IL-1, IL-6, and HGF levels and CTP scoring system imply that HGF secretion might be counteracting inflammatory response in liver tissue[77]. Molecular interaction between hematopoietic SCs and HGF has also been observed. HGF receptors show wide tissue distribution, but their distribution among hematopoietic SCs and reticuloendothelial cells attracts most attention. Together with G-CSF receptors, these receptors promote hematopoietic SC growth under *in vitro* conditions and are responsible for determining tissue macrophage

function in acute liver injury by affecting their IL-6 and IL-10 production[78,79]. The aforementioned theoretical and molecular knowledge along with the success achieved in mice seemed to be reasonable enough to justify the use of hematopoietic SCs for liver regeneration, but further research refuted this theory. Attempts at transplanting hematopoietic SCs into mice suffering from liver injury yielded poor results indicating an insufficient repopulation rate for appropriate tissue repair[80]. Genetic engineering of hematopoietic SCs could overcome the challenge of low tissue homing rates. Indeed, using genetically modified mesenchymal SCs expressing higher rates of HGF improves cell engraftment in liver injury[81].

In patients with AH treated by SC therapy (CD34+ and mesenchymal SCs, collected after mobilization with G-CSF), no significant improvement of liver function assessed by MELD score or proliferation of hepatic progenitor cells was noted in comparison to the control group[66]. Analysis of the liver tissue samples from patients with AH treated by SC therapy failed to show a difference in hepatocyte proliferative activity compared to the control group. However, the study showed expansion of macrophages together with an upregulated expression of genes involved in inflammation and regenerative pathways[67].

In view of the above, better understanding of the hematopoietic SC kinetics and genetic properties is needed to popularize their utilization in ALD.

Mesenchymal stem cells

Albeit being present in various tissues, mesenchymal SCs are mostly acquired from bone marrow, which embraces several different stem lineages. The most commonly used cell population is CD34+ endowed with potential to differentiate to hepatocytes, myocardial cells, pancreatic cells, *etc.*[58]. The plasticity of mesenchymal SCs appears to play a secondary role, since their immunomodulatory, anti-fibrotic and anti-oxidative properties spark more interest. In ethanol exposed mice, a simultaneous decline in IL-6 and TNF-□, and an increase in IL-10 and glutathione lays out their immunosuppressive effect[82]. In a murine model of colitis, adipocyte derived

mesenchymal SCs administered intravenously counterbalanced the decline in glutathione levels and superoxide-dismutase activity, thus preventing tissue injury caused by reactive oxygen species, which is seen in ALD as well[83].

Mesenchymal SCs orchestrate the overall inflammatory response of cells through complex alterations of cytokine networks, resulting in anti-inflammatory set up. While executive cells and their functions in the liver are various, probably the best course of events during injury can be seen by looking at macrophage polarization, a feature that encompasses liver tissue damage. M1 macrophage lineage is held responsible for triggering inflammation, while M2 Lineage contributes to down-regulation of inflammation and hepatocyte proliferation. M2 type macrophages secrete IL-10, VEGF, matrix metalloproteinases and TGF-β1, which are engaged in the resolution phase of liver damage. Although TGF-β1 is considered a proliferative agent, its long-term secretion during chronic liver disease restrains proliferation of hepatocytes together with IL-1 and interferon- secreted by M1 macrophages[84,85]. Apart from HGF, the impact on macrophage bipolar differentiation can also be listed as a key effect of mesenchymal SCs. At the cellular level, mesenchymal SC application hampers M1 macrophage and Th17 mediated inflammatory response, subsequently reducing the expression of TGF-β1 and fibrosis markers in carbon-tetrachloride induced liver fibrosis *in vivo*[55,86].

Interleukin-22

Several T-lymphocyte populations participate in the events occurring during ALD development. At the onset, a cascade of inflammatory events initiated by previously mentioned PAMPs and DAMPs is governed by Th1 Lymphocytes. In later stages, immune response becomes dichotomous. Th17 Lymphocytes represent the main subfamily associated with down-regulation of proinflammatory response and prevention of excessive tissue damage. Antagonistic effect of the Th17 subfamily is mediated by interleukin (IL)-22. IL-22 can bind to both IL-22 and IL-10 receptors

through which it sets off the STAT3 signaling pathway response. STAT3 pathway is also a target of IL-1 and IL-6; therefore, especially in chronic settings, this pathway can have both ameliorating and detrimental effects[87]. Moreover, the production of counteracting cytokines (IL-17 and IL-22) by the same lymphocyte type (Th17) shows the complexity of immune response during liver damage. In chronic conditions, Th17 Lymphocytes are located in parenchymal and extra-parenchymal tissue compartments and their count correlates with disease severity in humans[88]. On the other hand, in IL-22 knockout mice, hepatitis induced higher levels of liver enzymes indicating greater tissue damage in comparison to mice with normal IL-22 secretion[89]. Furthermore, IL-22 administration in a mouse model of ALD showed marked improvement in liver enzymes with reduction of liver steatosis. Notably, these hepatoprotective effects were abolished in STAT3 deficient mice[90]. In a murine model of ACLF, a shift from the pro-regenerative IL-6/STAT3 pathway to anti-regenerative IFN-ɣ /STAT1 pathway was noticed, with consequently impaired liver regeneration[91]. Treatment with IL-22 reversed this shift and improved outcomes by reprogramming impaired regenerative pathways[91].

All these studies implicated IL-22 as a possible therapeutic option for ALD, owing to its antioxidant, proliferative and antimicrobial effects, offering improvement of liver function with potentially few side effects. However, human studies on the use of IL-22 are still sparse. A study investigating an IL-22 analog, F-625, in the treatment of AH included 18 patients, divided in three groups according to the dose administered (10 μg/kg, 30 μg/kg, and 45 μg/kg). Significant amelioration of MELD score values, reduction in inflammatory cytokine concentrations, and better survival rates were observed after a 42-day follow-up period. More importantly, no serious adverse events were documented, showing IL-22 analogs as an attractive treatment modality that needs to be further investigated[92].

OTHER EXPERIMENTAL APPROACHES AND THERAPEUTICS WITH POTENTIAL BENEFIT IN THE TREATMENT OF SEVERE ALCOHOLIC HEPATITIS

Since treatment options for severe AH are limited, a number of other experimental approaches have been developed, based on the proposed mechanisms involved in the pathophysiology of AH[14,23]. Treatment targets of the potential experimental therapies are shown in Figure 1.

Anti-inflammatory agents

Considering AH as a severe inflammatory condition involving multiple mechanisms, cells and signaling pathways, anti-inflammatory agents represent an attractive treatment option.

Pentoxifylline is a phosphodiesterase inhibitor that has been used in the treatment of severe AH for its ability to inhibit TNF-α production. Although the initial randomized study showed improved short-term survival compared to placebo, these findings were not confirmed by subsequent studies, including the previously mentioned STOPAH trial[7,93]. Although it seems that pentoxifylline reduced the incidence of hepatorenal syndrome, the drug is considered unlikely to provide benefit and is not recommended for the treatment of severe AH by the European Association for the Study of the Liver (EASL) and the guidelines issued by the American Association for the Study of Liver Diseases, while the American College of Gastroenterology classifies pentoxifylline as a therapy with a potential benefit[3,5,6].

Treatment of severe AH with anti-TNF-α agents such as infliximab and etanercept seemed to be a desirable option, given their efficacy in the treatment of autoimmune diseases. Unfortunately, despite expectations, both drugs failed to show benefit in clinical trials for severe AH[14,23]. On the contrary, the use of TNF-α inhibitors was associated with an increased risk of severe infections and mortality, and therefore are not considered as a treatment option for severe AH by current guidelines[3,5,6]. The lack of their efficacy in AH may be explained by profound immunosuppression caused

by TNF blockage, but also by suppression of liver regeneration, which is also mediated by TNF-α and is necessary to restore liver function.

Other inflammatory cytokines have also been studied as therapeutic targets for severe AH, including IL-1 receptor antagonist anakinra, and a C-C chemokine receptor (CCR) 2 and 5 antagonist cenicriviroc, but data are still insufficient.

Antioxidants

Since increasing evidence suggests oxidative stress as one of the key mechanisms responsible for cellular injury in severe AH, the use of antioxidants has generated much interest.

N-acetylcysteine (NAC) is an antioxidant thought to restore glutathione stores and has been tested in several studies in the treatment of AH. However, the drug failed to show improvement in liver injury or survival. A combination of NAC with prednisolone showed greater early improvement in liver function, lower incidence of hepatorenal syndrome and infections, and improved short-term survival compared to prednisolone alone, but this effect was not sustained after 3 mo[94]. Further studies are awaited to confirm the efficacy of this combination[3,5,6].

Metadoxine is another antioxidant used in several small open-label studies, also in combination with a corticosteroid or pentoxifylline, where it showed improved survival. Along with some other beneficial effects and a favorable safety profile, metadoxine is an attractive option for the treatment of severe AH, but larger studies are expected to confirm these findings.

Gut dysbiosis and gut-liver axis

Gut-liver axis dysfunction with increased gut permeability and dysbiosis associated with alcohol drinking has been recognized as an important trigger for inflammation in AH. Consequently, a number of agents targeting gut barrier function and dysbiosis have emerged as treatment options for AH. These include bovine colostrum and hyperimmune bovine colostrum, antibiotics (rifaximin, amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, vancomycin, meropenem), probiotics (VSL#3, *Lactobacillus rhamnosus*) and fecal transplantation. Some of these treatments

have shown very promising preliminary results, and further studies are eagerly awaited.

Apoptosis

Alcohol induces hepatocyte death *via* necrosis and apoptosis processes, the latter including a number of pathways, which have been investigated as treatment targets in AH. Emricasan is a caspase inhibitor that showed promising results in early phase trials, but further studies were stopped because of concerns about liver toxicity. Selonsertib is another oral inhibitor of apoptosis with better safety profile, but its efficacy has not been established compared to corticosteroids.

Obeticholic acid

Cholestasis is one of the characteristic features of AH. Obeticholic acid is a farnesoid X receptor ligand that protects hepatocytes against bile toxicity. Favorable results in the treatment of cholestatic liver disease offer promises for obeticholic acid in the treatment of AH, pending results of ongoing studies.

DISCUSSION

Severe AH still represents a life-threatening condition with high short-term mortality. A lack of effective treatments with proven benefit and good safety profile imposes the need for new therapeutic approaches. Several pathways are being explored, targeting different mechanisms involved in the pathogenesis of AH. Stimulation of liver regeneration together with other beneficial effects suggest G-CSF and SC transplantation as possible therapeutic targets.

In animal models of toxic liver injury, G-CSF demonstrated evidence for increased mobilization of hematopoietic SCs, ameliorating liver injury, promoting liver regeneration, improved liver function and survival. These beneficial effects encouraged human studies, and the first results were promising, showing mobilization of SCs and proliferation of hepatic progenitor cells that were considered proof of efficacy[27]. Enhanced cell proliferation might not be crucial for the regeneration process as immunohistochemical staining using Ki67/CK+, chief markers of mitosis, showed

diverse results[27,67]. Subsequent randomized trials from India investigating the role of G-CSF treatment in patients with ACLF and AH demonstrated not only increased mobilization of SCs, but also improved clinical outcomes, measured by reduction in liver function scores, as well as improved survival[28,29]. Other positive outcomes included improved immune dysfunction with lower rates of complications, including hepatic encephalopathy, ascites, kidney injury, and infections[30,33,36,38].

However, in a large multicenter randomized study from Germany that enrolled 176 patients with ACLF, treatment with G-CSF did not improve liver function scores, occurrence of infections, or transplant-free or overall survival. Severe adverse reactions associated with the treatment, together with the lack of efficacy, led to premature termination of the study[39]. Discrepancies in the findings between this and previous smaller single-center studies (Table 1) are not completely understood. Several possible reasons for such conflicting results between Asian and European studies, including a selection bias or study design could not completely explain these differences[95]. Another possible explanation might be a large rate of infections in the German study that triggered an excessive inflammatory response, which could have counteracted the beneficial effects of G-CSF. However, the substantiated evidence from this trial strongly discourages the use of G-CSF in the treatment of ACLF. Still, it is important to note that the results of G-CSF for the treatment of ACLF cannot be directly extrapolated to AH due to great heterogeneity of ACLF cohorts regarding the cause of the underlying liver disease and the precipitating event for ACLF. In the previously mentioned German (GRAFT) study, alcohol abuse was the precipitating event in approximately half of the patients in both treatment groups, while bacterial infections and gastrointestinal bleeding were triggers for ACLF in the majority of the remaining cases [39]. However, the authors did not prove benefit of G-CSF over standard medical treatment in a cohort of patients with alcohol-related ACLF, which probably comprised mostly patients with AH.

Stem cell transplantation offers another possibility for improved liver regeneration. In recent years, a number of studies investigated the outcomes of SC

treatment in patients with liver disease of various etiology. However, studies focusing on patients with ALD are still sparse and recruited small numbers of patients; therefore, outcomes of each study are often insufficient to draw definitive conclusions. The majority of studies were actually phase I/II clinical trials, focused on the feasibility, safety and short-term outcomes of the procedure. One case-control study and five randomized controlled trials compared the effects of SC therapy to standard medical therapy alone (Table 2). The heterogeneity of the findings could be explained by several reasons.

Studies differed according to the SC population and terminology used, including peripheral blood SCs, mononuclear bone marrow SCs, mesenchymal SCs or hematopoietic SCs. Hematopoietic SCs are mononuclear cells typically collected by leukapheresis, and are often described as peripheral blood SCs. In studies where bone marrow aspiration was performed, mononuclear bone marrow SCs represent a combination of several SC types including mesenchymal cells that build up the stromal compartment of bone marrow. Except for mesenchymal SCs, the hallmark of the aforementioned cells is expression of transmembrane phosphoglycoprotein, CD34. In a small study, two CD34+ cell populations were differentiated based on their *in vitro* property of adherence. Adherent CD34+ cells expressed genes important for hepatic tissue differentiation; therefore, SCs with specific phenotype could be pivotal for gaining therapeutic effect, although they were encountered in a relatively small fraction after mobilization[58]. Promising results were obtained in a small study using G-CSF mobilized mononuclear CD34+ peripheral blood SCs collected by leukapheresis and injected *via* hepatic artery after *in vitro* cultivation for 7 days[62]. It could be assumed that a 5-fold increase in cell count obtained by *in vitro* cultivation could yield better results.

Except for small and heterogeneous patient groups with different conditions (alcoholic cirrhosis, AH, and some with multifactorial origin of liver disease), studies used different G-CSF dosing regimens and SC administration routes (*via* hepatic artery or portal vein), number of SC infusions, outcomes measures, and follow-up period.

In the study including 10 patients, 4 of them with AH, having undergone bone derived mononuclear cell transplantation, serum bilirubin and INR levels decreased and albumin levels increased after 4-month follow-up[61]. In another study including two patients with AH, histological analysis did not reveal changes in fibrosis stage; however, improvement of both CTP and MELD scores was achieved[60]. In a controlled trial, patients with decompensated ALD treated with G-CSF mobilized mononuclear bone marrow cells had reduction in steatosis and improved MELD scores after 12-week follow-up, but similar to patients treated by standard medical therapy[66]. There was no difference between the SC treated and control groups according to hepatocyte proliferation. However, a more pronounced macrophage infiltration of the liver was observed in the treatment group, together with higher expression of the genes involved in regenerative processes[66,67]. Another small study involving two patients with AH also assessed CTP and MELD scores after hematopoietic SC aspiration and administration, but without G-CSF stimulation[64]. At the end of a 12-month follow-up period, no significant differences in serum albumin and bilirubin levels or mean CTP and MELD scores were noticed. Using scintigraphy in this study, the authors managed to document a high percentage of SC retention in liver tissue (41% and 32% after 3 and 24 h, respectively). This research is valuable in terms of assessing cell tropism and dissemination, but also reported several major adverse events, raising concerns about the safety of the procedure[64]. The effects of SC treatment on liver fibrosis were also investigated in several studies, but benefits were shown in only one of them[71]. Probably the most discouraging results were obtained in a randomized multicenter study from the United Kingdom, investigating the efficacy of G-CSF treatment alone or in combination with CD133+ hematopoietic SC infusion obtained by leukapheresis, and compared to standard medical treatment in patients with compensated liver cirrhosis[96]. No improvement of liver dysfunction as assessed by MELD score or fibrosis was observed in either treatment group when compared to standard medical care. However, treatment with G-CSF alone or in combination with SC therapy was associated with an increased frequency of adverse events[96].

The explanation of this paradox and lack of evidence for clinical efficacy of SC therapy for the treatment of AH is still not completely understood. One of the possible explanations might be a relapse of alcohol use in the follow-up period, which is common in clinical practice. In the STOPAH trial, only 45% of patients with AH abstained from alcohol at six months and 37% at 12 mo, meaning that relapse of alcohol use occurred in two thirds of patients surviving an episode of AH after a one-year follow-up period[7]. In a study from Barcelona investigating alcohol abstinence in patients surviving an episode of AH, the investigators also report complete abstinence in only 39% of patients after a median follow-up period of 55 mo, and showed that it had positive impact on long-term survival[97]. Similar results were obtained in a study from the United Kingdom, where 65% of patients surviving an episode of AH experienced alcohol relapse, and abstinence was shown to be the only predictor of long-term survival in these patients[98]. Indeed, in a previously mentioned randomized study investigating bone marrow mononuclear cell transplantation with G-CSF in patients with decompensated ALD, 81% of whom met the histological criteria for AH, alcohol abstinence was achieved in 67% of patients during 12-week follow-up period, while almost one-third returned to moderate alcohol drinking [66]. The study failed to show benefit of such therapy compared to standard medical treatment, and alcohol relapse in 31% of study patients might be one possible explanation for such results. Another possible explanation is that the effects of SC and other treatments aimed to stimulate liver regeneration depend on the etiology of the liver disease. It has been shown that in ALD related cirrhosis, acute and chronic exposure to alcohol interferes with liver regeneration capacity and may actually impair hepatic progenitor cell response to injury, as mentioned previously, resulting in poor outcomes[14,23,99,100].

CONCLUSION

Ineffective liver regeneration is probably one of the key contributors to liver failure in patients with severe AH. Many cytokines, including TNF-α, IL-6 and IL-22, and STAT3 and NFκB pathways have been shown as potent activators of hepatic

regeneration. However, an altered differentiation process seems to cause repopulation of the liver parenchyma by immature hepatocytes that fail to differentiate into mature hepatocytes, which could explain why some patients with severe forms of AH fail to recover. Novel treatment options that would help overcome this obstacle and improve liver regeneration seem intriguing, but so far, the evidence for their efficacy is unconvincing.

The lack of a reliable animal model that could represent the whole spectrum of liver changes associated with ALD in humans still is one of the major obstacles in the research of ALD. Development of an experimental model including not only steatosis and mild inflammation, but also fibrosis, severe inflammation and bilirubinostasis with an altered regeneration process, which are the hallmarks of severe AH in humans, represents an imperative for future research and development of new treatment options.

Better understanding of the molecular mechanisms behind ALD has given us apprehension of the multifaceted nature of the disease revealing novel treatment targets. In the light of evidence from recently published studies, G-CSF therapy does not seem like a feasible treatment option in the near future. Succeeding studies on SC therapy should aim to investigate long-term effects and define precise SC recruitment protocols providing clinicians with clear guidelines on both the G-CSF application regimen and SC administration routes. Until further insights, future research on liver reconstitution remains a challenge.

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