CHAPTER 1

Introduction and aim of the thesis

Danial Afsharzadeh¹
Martin C. Harmsen²
Klaas Nico Faber¹,³

Departments of ¹Hepatology and Gastroenterology, ²Pathology and Medical Biology and ³Laboratory Medicine, Center for Liver, Digestive and Metabolic Disease, University of Groningen, University Medical Center Groningen Groningen, The Netherlands.
1.1 INTRODUCTION

Liver disease is a significant health problem and accounts for over two million deaths per year worldwide\(^1\text{-}^4\). In clinical practice, liver disease is divided into acute and chronic forms, based on the (initial) presentation and persistence of liver injury. However, this classification is an oversimplification of the broad spectrum of the different types of liver diseases and chronic liver injury is, in part, the result of extended acute liver injury that has been continued over time\(^5,^6\). Still, chronic liver diseases are accompanied by additional pathological processes when compared to acute forms, which may require different therapeutic approaches. Acute liver damage is typically induced by viruses or toxins and is characterized by hepatocyte death that can lead to significant impairment of liver function and structure\(^7\). The mode of hepatocyte death can be either through necrosis, apoptosis or mixed variants thereof, and is determined by the type of damage, etiology, duration and extent of liver injury. Acute liver injury can progress to acute liver failure (ALF), which is a critical condition with a mortality rate of more than 80\%\(^8\). Drug intoxication is considered as the leading cause of ALF and acetaminophen (APAP) overdose represents the most common cause of drug-induced ALF worldwide\(^9\). APAP-induced ALF may occur after taking a large single oral dose of APAP (\(>7,500\) mg) or after higher than recommended doses for several consecutive days\(^9,^10\). Viral infection, with hepatitis A, B and E, is another common cause of ALF. Autoimmune hepatitis, metabolic disease, non-viral infection and cancer are among less frequent causes of ALF\(^9\). Loss of functional liver mass triggers the liver to regenerate, a remarkable feature of this organ. Patients may survive loss of over 50\% of liver function and, if the cause of liver injury can be eradicated, the liver will regain to its original volume in several weeks. Thus, ALF only occurs when there is a misbalance between the disease-induced liver damage and the capacity of the liver to regenerate. The liver regeneration process comprises the proliferation of remaining healthy hepatocytes as well as activation, proliferation, differentiation and maturation of hepatic progenitor cells, thereby enabling restoration of the hepatic function and architecture\(^11,^12,^12\). Chronic liver injury is a result of continued hepatocyte injury and death, which may slowly develop to liver failure over many years. Damaged hepatocytes release reactive oxygen species (ROS) and inflammatory mediators, such as TGF-\(\beta\)1, TNF-\(\alpha\), EGF and IGF that activate liver resident macrophages (Kupffer cells), recruit circulating macrophages and inflammatory T-cells. While this is a primary response to clear possible infections and initiate liver regeneration, persistent activation of these cells also leads to the activation of liver fibroblasts\(^13\), particularly hepatic stellate cells (HSC) and portal myofibroblasts.
Introduction and aim of the thesis

(PMF). In normal physiology, HSC are quiescent (qHSC) in the (healthy) liver, where these play a central role in controlling systemic vitamin A homeostasis. Upon liver injury, qHSC transdifferentiate to migratory and proliferative myofibroblasts (activated HSC; aHSC), a process in which the lose their vitamin A stores. The activated liver fibroblasts, both aHSC and PMF, produce and deposit extracellular matrix proteins (ECM), in particular collagen, fibronectin and laminin, in the liver, required for the healing of the damaged tissue. In a normal

Figure 1. Spectrum of non-alcoholic fatty liver disease (NAFLD). Non-alcoholic fatty liver disease (NAFLD) is a spectrum of diseases ranging from fatty liver (simple steatosis), non-alcoholic steatohepatitis (NASH) and NASH-associated fibrosis and cirrhosis, which predisposes for the development of hepatocellular carcinoma.
repair process, any excessive ECM is degraded by specific matrix metalloproteases (MMPs) and normal liver architecture is restored. However, persistent damage causes a misbalance in ECM production and turnover, because degradation is often insufficiently induced or even decreased. The excessive ECM deposition leads to formation of interstitial scar tissue, which is called fibrosis. Over time, fibrosis can spread throughout most of the liver, destroying the internal structure and impair liver regeneration and thereby reducing liver function. Such severe and irreversible scarring of the liver is called cirrhosis. Cirrhosis causes increased intrahepatic resistance to blood flow and portal hypertension, as well as hepatic insufficiency. Advanced cirrhosis is a risk factor for developing hepatocellular carcinoma and most of the morbidity and mortality related to liver disease occur after cirrhosis develops.

In a global perspective, non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease affecting approximately 25% of the world population, in particular in Westernized countries. As the name implies, NAFLD is characterized by excessive fat accumulation in the liver, especially triglycerides and cholesterol, without a clear relationship with alcohol intake. Obesity is the main trigger for NAFLD, which is actually a spectrum of liver diseases, ranging from simple steatosis, non-alcoholic steatohepatitis (NASH) to NASH-associated fibrosis and cirrhosis that predisposes for hepatic carcinogenesis. In this spectrum, NASH distinguishes from simple steatosis by the presence hepatic inflammation, which may co-exist with different stages (F0-F4) of fibrosis. Hepatocyte damage and inflammation play leading roles in disease progression, amongst others via activation of liver fibroblasts and the resultant fibrosis. Even though only 20% of patients with NAFLD also meet the criteria for NASH, the latter condition is being considered the major cause of liver fibrosis and cirrhosis in patients with chronic liver disease (see Figure 1).

Treatment options for patients with end-stage liver disease are still extremely limited, and liver transplantation remains as the only life-saving therapy. However, scarce availability of donor organs limit the number of patients who can benefit from this treatment. Moreover, liver transplantation remains a risky procedure, associated with high health care expenses, risks of complications related to the surgery, and the necessity for lifelong immunosuppression, which negatively impact the patient’s quality of life. Thus, there is an evident need for alternative therapeutic approaches for liver disease patients that otherwise can only be helped by liver transplantation. Therapies using mesenchymal stromal cells (MSC) have recently emerged as being one of the most promising options for patients with chronic liver disease, being a less aggressive procedure and potentially equally curative as liver transplantation. MSC encompass a population
of undifferentiated cells\textsuperscript{24} that reside in virtually all tissues of the body, including bone marrow\textsuperscript{25}, umbilical cord\textsuperscript{26} and adipose tissue\textsuperscript{27}. As a common feature, MSC are able to differentiate \textit{in vitro} into cartilage, adipocytes and osteogenic cells. The yield of MSC isolated from different tissues varies considerably, between relatively low from bone marrow and high (around hundred million per liter) from fat tissue. The ease of obtaining subcutaneous adipose tissue by a minimally invasive method is a clear advantage of adipose tissue-derived mesenchymal stromal cells (ASC) over MSCs from other tissues\textsuperscript{29-31}. Demonstration of clinical feasibility and

\textbf{Figure 2. Chemokine receptors in MSC.} MSC chemokine receptors are represented in blue. Chemokines able to stimulate MSC are beside their respective receptors (in green).
therapeutic efficacy of MSC in the past two decades have opened doors for a large number of clinical trials applying MSC in a variety of common and life-threatening diseases, such as stroke\textsuperscript{32,33}, heart failure\textsuperscript{34}, COPD\textsuperscript{35} and liver failure\textsuperscript{36}. Some trials have indicated that the route of administration of MSC is a determining factor for their therapeutic efficacy\textsuperscript{37,38}, but to date, no Gold Standard route of MSC delivery has been established. Several studies proposed that systemic infusion of MSC is the preferred route of administration\textsuperscript{39-41}, but improving tissue-specific homing of MSC is still one of the major challenges in MSC-based therapies. Indeed, various imaging studies show that only a small percentage of intravenously administered MSC reach the target tissue\textsuperscript{42-44}. A growing body of evidence ascribes this low efficiency to the limited expression of homing molecules, \textit{i.e.} chemokine receptors, during the \textit{in vitro} expansion of MSC\textsuperscript{45,46}. MSC are shown to be attracted to the chemical gradient of chemokines which are being released at the site of injury\textsuperscript{47-49}. Chemokines are small secreted proteins ranging in size from 7 to 13 kDa. The arrangement of four amino terminal cysteines is used to group chemokines into structurally related families. Accordingly, four families of chemokines have been defined: the CCL family, the CXCL family, the CL family and the CX3CL family\textsuperscript{50}. Similarly, chemokine receptors are being categorized based on their interaction with specific chemokine families. Thus, CXCR receptors bind CXC chemokines, and CCR receptors bind CC chemokines. However, as there are fewer chemokine receptors than chemokines, a single chemokine receptor may bind several different chemokines, and a single chemokine also may bind more than one receptor\textsuperscript{47,50} (see Figure 2).

MSC administration has been shown to be beneficial to treat patients with end-stage liver disease in several studies, as evidenced by improved liver function, reduced hepatocyte apoptosis, enhanced hepatocyte proliferation, and most importantly, increased survival of patients with severe liver disease\textsuperscript{23,51,53,54}. Nevertheless, the clinical application of MSC holds multiple safety and ethical concerns that call for more in-depth investigation to define the factors that actually determine their therapeutic effect\textsuperscript{55}. A growing body of evidence indicates that MSC-derived extracellular vesicles (EVs) are main carriers of the therapeutic factors and largely mirror the phenotype of their parent MSC\textsuperscript{56,57}. Such MSC-derived EVs could be the basis of a novel cell-free therapy for end-stage liver disease. EVs are released from a variety of cells (including MSC) and are classified into microvesicles (MVs), exosomes, and apoptotic bodies, according to various morphological (size and shape) and biochemical parameters\textsuperscript{58,59}. EVs contain proteins, lipids and nucleic acids, which they can transport to specific tissues, as they can be captured by other cells via variety of ways, such as direct membrane
Introduction and aim of the thesis

Figure 3. Mesenchymal stromal cell derived-extracellular vesicles (MSC-derived EVs) rescue the liver damage and ameliorate liver fibrosis. Chronic liver damage mediates by progressive hepatocyte injury which spans for a long time. Damaged hepatocytes release reactive oxygen species (ROS) and inflammatory mediators, such as TGF-β1, TNF-α, EGF and IGF, inducing the recruitment of inflammatory cells and resulting in accumulation of activated liver fibroblasts, particularly hepatic stellate cells (HSC) and portal myofibroblasts (PMF). Activated liver fibroblasts facilitate synthesizing large amounts of extracellular matrix proteins (ECM) which causes tissue fibrosis. MSC derived-EVs are able to rescue the liver damage and ameliorate liver fibrosis through alleviating hepatocyte damage and regression of activation in liver fibroblasts.
fusion, receptor mediated fusion or endocytosis or a combination\textsuperscript{60-62}. So far, MSC-derived EVs have shown multiple therapeutic effects, including in tissue regeneration, wound healing and immunomodulation\textsuperscript{63}. Importantly, MSC-derived EVs have been shown to be less immunogenic and, compared to MSC, show a lower risk of allogenic immune rejection by the host\textsuperscript{63,64}. Small non-coding microRNA molecules (miRNAs, mostly 22 nucleotides long) have recently gained extra attention amongst EV-cargo, and a rapidly increasing number of reports attribute the therapeutic potential of MSC-derived EVs to miRNA transfer to affected cells of injured organs\textsuperscript{65-67}. The therapeutic benefits of MSC-derived EVs are being examined in a range of acute and chronic liver disease\textsuperscript{68,69}. As it appears, MSC-derived EVs are capable of alleviating hepatocyte damage and suppress the activation of liver fibroblasts (see Figure 3). Still, the therapeutic mechanisms of MSC-derived EVs are very much unexplored and need further investigation.
1.2. THE AIM AND OUTLINE OF THE THESIS

The overall aim of this thesis is to investigate the therapeutic potential of extracellular vesicles from human adipose-derived stromal cells (hASC-derived EVs) in the treatment of acute and chronic liver disease. In Chapter 2, we focused on the trafficking of hASC migration to injured and early-fibrotic liver tissue, ex vivo. We tested the migration potential of hASC to human precision-cut liver slices (PCLS). In order to distinguish the migratory signal in the fibrotic tissue, we analyzed the hASC migration towards hepatocytes, quiescent hepatic stellate cells (qHSC) and activated hepatic stellate cells (aHSC), separately. Moreover, using specific inhibitors of the chemokine receptors CXCR2, CXCR3 and CXCR4, we investigated the mechanisms controlling the migration of hASC to the fibrotic liver. In Chapter 3, we focused on the therapeutic capacity of hASC-derived EVs in liver fibrosis. Activation of hepatic fibroblasts is the hallmark for the onset of liver fibrosis. We first evaluated whether hASC suppress activation and proliferation of liver fibroblasts in a paracrine manner. As this was the case, we next evaluated the potential of hASC-derived EVs and EV-free conditioned medium to suppress activation and proliferation in liver fibroblasts in vitro, as well as in vivo mouse model of liver fibrosis and in a human ex vivo using human precision-cut liver slices. In addition, we analyzed the miRNA content of hASC-derived EVs with a focus on miRNAs with anti-fibrotic activities. In Chapter 4, we investigated the potential of hASC-derived EVs in alleviating acute liver damage in mice exposed to a single high dose of paracetamol (APAP) or CCl₄. Both the prophylactic and the therapeutic potential of hASC-derived EVs to prevent liver injury were evaluated. We analyzed liver histology and serum markers of liver damage, as well as markers of hepatic inflammation and early markers of fibrosis. In Chapter 5, we studied the potential of hASC-derived EVs to ameliorate simple steatosis in mice, being the first stage in the development of NAFLD. Mice were given a high fat-high cholesterol diet for 6 weeks and treated with hASC-derived EVs in the final 3 weeks. We evaluated body and liver weights of the mice and analyzed liver histology and serum markers of the liver damage. Moreover, we determined whether hASC-derived EVs affect Western-diet induced cholesterol and triglyceride levels in liver and in serum. Finally, transcript analysis was performed to reveal potential miRNAs involved in the hASC-EV-induced effects. In Chapter 6, we summarize the results obtained in the experimental studies of this thesis and provide an outlook for future directions of hASC-derived EV therapy for patients with liver disease.
REFERENCES

Introduction and aim of the thesis


