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Hepatic Myofibroblasts in Liver Fibrogenesis

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1. Introduction

On a worldwide perspective chronic liver diseases (CLDs) are very common pathologic conditions which are characterized by reiteration of hepatocyte injury that is mainly induced by chronic infection by hepatitis B and C viruses (HBV and HCV), autoimmune injury and metabolic and/or toxic/drug – induced causes, with chronic alcohol consumption being predominant particularly in western countries. Chronic liver injury is reported to result in the chronic activation of both inflammatory and wound healing response that, in association with other major pathogenic mechanisms (oxidative stress, derangement of epithelial-mesenchymal interactions and possibly epithelial to mesenchymal transition, see later in section 3), can sustain persistent liver fibrogenesis (i.e., the process) and represent the prominent driving force for liver fibrosis (i.e., the result) (Parola and Robino, 2001; Friedman 2003; Bataller and Brenner, 2005; Friedman 2008b; Novo and Parola, 2008; Parola et al., 2008).

Literature data from the last two decades indicate that hepatic fibrogenesis in a CLD has to be envisaged as a dynamic and highly integrated molecular, tissue and cellular process that, irrespective of the aetiology, leads to the progressive accumulation of extracellular matrix (ECM) components in an attempt to limit hepatic injury. Persistent liver fibrogenesis is currently considered as the critical process responsible for the progression of any CLD to the end-points of liver cirrhosis and hepatic failure. According to current definition, cirrhosis should be regarded as an advanced stage of fibrosis, being characterized by the following major features: a) the formation of regenerative nodules of hepatic parenchyma surrounded and separated by fibrotic septa; b) the development of significant changes in organ vascular architecture, portal hypertension and related clinical complications (i.e., variceal bleeding, hepatic encephalopathy, ascites and hepatorenal syndrome) (Friedman, 2003, 2004; Pinzani and Rombouts, 2004; Bataller and Brenner, 2005; Friedman 2008b).

In any CLDs fibrotic progression can proceed through at least four distinct patterns of fibrosis that have close relationships with the underlying aetiology and are also related to the "topographic site" of tissue injury, the involvement of different populations of myofibroblasts (MFs) and the predominant pro-fibrogenic mechanism (Cassiman and Roskams, 2002; Pinzani and Rombouts, 2004; Parola et al., 2008; Parola and Pinzani, 2009).

1.1 Bridging fibrosis

This pattern of ECM deposition and fibrotic septa formation is typically described in the liver of patients carrying HBV- or HCV-related chronic hepatitis. As a result of portal-central bridging necrosis the pattern is characterized by the formation of a) portal-central fibrotic septa, that connect portal areas with the area of central vein, b) portal-portal fibrotic septa, that connect distinct portal areas, as well as of c) blind fibrotic septa in the parenchyma. As any pathologist may easily recall, to this pattern belong classic histopathological images of fibrotic septa leading to the obliteration of central veins and of early changes in vascular architecture and connections with the portal system, events that with the time favor the development of portal hypertension. The pattern of bridging fibrosis recognizes the chronic activation of wound healing as the major pathogenic mechanism driving fibrosis progression and, as detailed later in this chapter, fibrogenesis is predominantly sustained in these settings by hepatic populations of pro-fibrogenic MFs that originate mainly from hepatic stellate cells (HSCs) or, to a less extent, from either portal fibroblasts or from bone marrow - derived stem cells. Extensive literature data also suggest that oxidative stress and reactive oxygen species (ROS) offer an additional major contribution in sustaining liver fibrogenesis in this pattern of bridging fibrosis.

1.2 Perisinusoidal / Pericellular fibrosis

This specific pattern of fibrosis has been described in CLDs which follow either excess alcohol consumption (ASH or alcoholic steatohepatitis) or metabolic derangement and then progression from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH). In these clinical settings, which are increasingly common particularly in western countries, excess deposition of ECM components is first detected in the space of Disse leading to the peculiar "chicken-wire" pattern. MFs originating from the activation of hepatic stellate cells (HSC/MFs) are believed to represent the most relevant pro-fibrogenic cellular effectors in these conditions, with ROS and oxidative stress playing a predominant pro-fibrogenic and pathogenic role.

1.3 Biliary fibrosis

This is a pattern which in humans is typically detected in a number of conditions affecting the biliary tree (primary biliary cirrhosis, primary sclerosing cholangitis, secondary biliary cirrhosis) that are characterized by a peculiar scenario involving concomitant proliferation of bile ductules and periductular MFs. In these clinical settings MFs mainly derive from periportal fibroblasts or, as recently suggested, by epithelial to mesenchymal transition (EMT) of cholangiocytes, an issue that at present is controversial and highly debated (see section 3.4). In any case, the biliary fibrosis scenario is dominated by the formation of portal-portal fibrotic septa that in human patients for long time do not significantly affect vascular connections with the portal system. Major pathogenic mechanisms sustaining this pattern of fibrosis have been identified either in alterations in the interactions between cholangiocytes and mesenchymal cells (with cholangiocyte transition into MF-like phenotype being still debated) or in alterations of redox equilibrium (i.e., oxidative stress).

1.4 Centrilobular fibrosis

Although this pattern is commonly included in the classification, centrilobular fibrosis is indeed unrelated to CLDs but, rather, is typically observed in those patients being affected by chronic heart failure: in these patients a significant alteration of venous outflow is realized that, with the time, leads to the formation of fibrotic septa developing among central vein areas (central-central septa) which, in turn, result in the unique scenario which is often described as of "reversed lobulation".

2. Hepatic myofibroblasts

Hepatic myofibroblasts (MFs) represent a heterogenous population of pro-fibrogenic cells, mostly positive for α–smooth muscle actin (α-SMA), that are mainly found in chronically injured livers (i.e., fibrotic and or cirrhotic) (Cassiman et al., 2002; Friedman, 2008a, 2008b; Parola et al., 2008; Parola and Pinzani, 2009) and, irrespective the specific aetiology of a CLD and of the prevalent pattern of fibrosis, sustain liver fibrogenesis in addition to injured hepatocytes, activated Kupffer cells and sinusoidal endothelial cells. Hepatic populations of MFs share a number of common properties which include high proliferative attitude, the ability to contract in response of vasoactive mediators, and the ability to actively participate to CLD progression by means of their multiple phenotypic responses. This includes excess deposition of ECM components and ECM altered remodelling as well as the synthesis and the release (paracrine/autocrine) of critical polypeptide mediators which sustain and perpetuate fibrogenesis, chronic inflammatory response and angiogenesis. Along these lines, hepatic MFs can be envisaged as a unique and crucial cellular crossroad where incoming paracrine and autocrine signals, including growth factors, inflammatory and angiogenic signals, chemokines, adipokines, as well as ROS, are integrated in order to "operate" phenotypic responses designed to sustain fibrogenesis and the progression of CLDs to the end-points of cirrhosis and hepatic failure.

As recently reviewed (Parola et al., 2008; Parola & Pinzani, 2009) progressive fibrogenesis is believed to be sustained by four main pro-fibrogenic mechanisms: a) chronic activation of the wound healing response, a mechanism that applies virtually to any CLDs and predominates in HBV or HCV chronic injury or autoimmune liver diseases; b) oxidative stress, with the generation of ROS and other oxidative stress – related reactive mediators, a mechanism that again applies to all CLDs but is predominant in either alcoholic liver disease (ALD) and NASH; c) derangement of epithelial-mesenchymal interactions, as detected in chronic cholangiopaties and, more generally, all the conditions of biliary fibrosis; d) EMT.

Some years ago it has been proposed a classification of the different hepatic populations of MFs which is based on the specific antigen profile and the tissue localization of these pro-fibrogenic cells in the context of a chronically injured liver (Cassiman et al., 2002). This classification recognizes at least three different kind of MFs.

2.1 Portal/septal MFs (PS/MFs)

These MFs are believed to originate mainly from portal fibroblasts (i.e., fibroblasts residing in the connective tissue of portal areas in normal conditions) through a process of

activation/transdifferentiation. PS/MFs are typically detected in the expanded connective tissue around portal tracts (portal MFs) or in the inner part of fibrotic septa (septal MFs). Irrespective of their localization, they share a common and overlapping antigen repertoire that allows their recognition "in vivo". The antigen profile of human PS/MFs includes, in addition to α-SMA, expression of glial fibrillary acidic protein (GFAP), brain-derived nerve growth factor (BDNGF) and α-B-crystallin (ABCRYS).

2.2 Interface MFs (IF/MFs)

Interface MFs are α-SMA – positive cells detected at the edge between fibrotic septa and the surrounding parenchyma (i.e., where active fibrogenesis occurs). These cells can originate from activation/transdifferentiation of hepatic stellate cells, portal fibroblasts as well as from bone marrow – derived mesenchymal stem cells (Russo et al., 2006; Valfrè di Bonzo et al., 2008; Forbes & Parola, 2011) following their engraftment into chronically injured livers. Human IF/MFs express all the typical markers already mentioned (α-SMA, GFAP, BDNF, ABCRYS) as well as nerve growth factor (NGF), neural cell adhesion molecule (N-CAM) and neurotrophin-4 (NT-4).

2.3 Activated, MF-like, hepatic stellate cells (HSC/MFs)

HSC/MFs are α-SMA-positive cells found primarily in or around capillarised sinusoids of fibrotic/cirrhotic livers that by definition originate only through a process of activation/differentiation from hepatic stellate cells (HSC), a kind of cells which have their physiological location in the space of Disse and in normal conditions are believed to store vitamin A and retinoids, to contribute to the synthesis of the local ECM components as well as to act as liver specific pericytes. HSC/MFs are positively stained "in vivo" for α-SMA and can be recognized from other MFs for their positivity to a list of additional markers which includes neurotrophin-3 (NT-3), tyrosine kinase B or C (Trk-B, Trk-C), synaptophysin and p75, the low-affinity nerve growth factor receptor.

3. The multiple origin of hepatic myofibroblasts

A fascinating and still incompletely resolved issue is represented by the "in vivo" origin of hepatic MFs. According to current literature hepatic MFs mainly originate from hepatic stellate cells or from fibroblasts of portal areas through a process of activation and trans-differentiation. In addition, hepatic MFs have been reported to originate also from bone marrow – derived stem cells, including mesenchymal stem cells or circulating fibrocytes, able to engraft chronically injured liver (Forbes & Parola, 2011). It has also been proposed that myofibroblasts or pro-fibrogenic cells may originate from either hepatocytes or cholangiocytes through a process of EMT, an issue that is at present highly controversial and debated (see section 3.4). Although hepatic MFs may then originate from different sources the interested reader should note that these cells are likely to display the same pro-fibrogenic properties and phenotypic responses. Moreover, it has been proposed that HSC/MFs may play a critical role in the modulation of immune responses in CLDs and to strictly interact with either hepatic progenitor (stem) cells and/or with malignant cells of primary hepatocellular carcinomas or of metastatic cancers. In the following sections we will briefly summarize major information on the different intra- and extra-hepatic origin of MFs.

3.1 Hepatic stellate cells as a major intrahepatic source of MFs

Under physiological conditions HSC are perisinusoidal cells of still uncertain embryological origin which are responsible for at least four main functions: a) HSC are responsible for the synthesis of basal membrane like – ECM components of the subendothelial space of Disse where they are located; b) HSC are responsible for the storage and the metabolism of vitamin A and retinoids (HSC represent the main site of storage for these compounds in mammalians); c) HSC act as "liver specific pericytes" taking intimate contacts with liver sinusoidal endothelial cells (LSEC); d) HSC contribute significantly to hepatic development and regeneration following either acute liver injury of liver resection (Friedman 2008a).

HSC have been the first cell source of pro-fibrogenic MF-like cells to be identified and most of published studies performed on the pathogenic mechanisms of liver fibrosis deal with HSC/MFs. For these cells Scott Friedman's laboratory has proposed the two-step process of activation and trans-differentiation, i.e. the process that leads HSC to acquire the MF-like phenotype (Friedman, 2003, 2008a). HSC-MFs are currently believed to be involved in most of clinical conditions of CLDs, with a predominant involvement in the pattern of fibrosis progression defined as "perisinusoidal/pericellular fibrosis", recognising a metabolic or alcoholic aetiology. HSC contribute significantly also to the origin of IF/MFs and to the pattern of "bridging fibrosis" found in patients affected by chronic viral hepatitis (Friedman, 2008a,b; Parola et al., 2008).

Experimental and clinical studies dealing with HSC and HSC/MFs have been fundamental for our present knowledge concerning liver fibrogenesis and related molecular mechanisms by revealing that, under conditions of chronic liver injury, quiescent HSC located in the perisinusoidal space of Disse can undergo a peculiar process of activation. Human as well as rodent HSC undergo "in vitro"changes in morphology and operate phenotypical responses in their trans-differentiation from the original "storing or quiescent phenotype" to the one of activated MFs. The activated MF-like phenotype includes the following relevant pro-fibrogenic features (Friedman 2008a; 2008b; Pinzani and Marra, 2001; Pinzani and Rombouts, 2004; Bataller and Brenner, 2005; Parola et al., 2008): a) a high proliferative attitude in response to growth factors and other mediators; b) an increased ability to synthesize ECM components, mainly fibrillar collagens (i.e., collagen type I and type III), as well as of factors involved in ECM remodelling; c) the ability to migrate in response to chemoattractants; d) the property to produce and release growth factors (autocrine loops) and pro-inflammatory cytokines; e) the ability to contract in response to vasoactive compounds. All these changes and phenotypic responses are considered to be similar to those occurring "in vivo" and to represent a functional paradigm common to all pro-fibrogenic MFs, whatever the origin (Friedman 2008b; Parola et al., 2008; Parola and Pinzani 2009; Forbes & Parola, 2011).

3.2 Portal fibroblasts as an emerging additional intrahepatic source of MFs

Portal fibroblasts (PFs) in the normal liver are α-SMA positive cells characterized by a morphological phenotype and by an antigen repertoire which are close to those expressed by other fibroblasts. Moreover, PFs express the highly specific fibroblast marker TE7, that is not expressed by other potential cellular sources of MFs like HSC (Dranoff & Wells, 2010), as well as other rather specific markers including IL-6, fibulin 2, elastin and the ecto-ATPase

nucleoside triphosphate diphosphohydrolase-2 (NTPD2). The origin of PFs is still uncertain and these cells may alternatively originate either from α-SMA positive cells of the ductal plate during human embryogenesis or, as recently proposed by a murine study, from a putative precursor in the early embryo development able to give also raise to HSCs (Asahina et al., 2009).

According to current literature PFs undergo myofibroblastic differentiation in the chronically injured liver and when cultured on plastic or glass. Portal myofibroblasts (P/MFs), like typical myofibroblasts, express large numbers of α-SMA–containing microfilament bundles arrayed in parallel to the long axis of the cell. At present no definitive evidence has been presented to establish whether P/MFs can proliferate and can be passaged in culture or whether they can revert to a non MF state either "in vitro" or "in vivo", as shown in the past for HSC and HSC/MFs.

Accumulating evidence suggest that PFs as well as P/MFs have a relevant role in liver fibrogenesis. In particular, evidence for the pro-fibrogenic role is unequivocal in experimental conditions of biliary fibrosis such as those from experimental models of cholestatic injury, namely the model of bile duct ligation (BDL) in rodents as recently reviewed (Dranoff and Wells, 2010). The first studies reported that PFs start to deposit matrix in portal areas following BDL before undergoing myofibroblastic differentiation; moreover, PFs proliferate very rapidly around bile ductular cells following BDL and desmin-negative MFs (not originating from HSC) appear early (i.e., within 48 hrs) after BDL adjacent to the proliferating ductules. More recent studies provide evidence suggesting that the pro-fibrogenic role of PFs and P/MFs in biliary fibrosis mostly relies on the fact that the injury to bile duct epithelial cells (BDEC) is a prerequisite for the differentiation of PFs into P/MFs. The elegant and now validated hypothesis is that, once damaged, BDEC start to express transforming growth factor β2 (TGFβ2) and release a number of growth factors and pro-inflammatory mediators, including platelet-derived growth factor -BB (PDGF-BB), interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1 or CCL2), that can be responsible for differentiation of PFs (that express related receptors) towards the P/MF phenotype. This process closely resemble the process of HSC activation and even more relevant, emerging evidence indicate that the acquisition of the P/MF phenotype is apparently followed by a process of perpetuation in which P/MFs start to behave like HSC/MFs.

An emerging issue is that P/MFs may significantly contribute to CLD progression not only in biliary fibrosis but also in other clinical conditions characterised by bridging fibrosis irrespective of the aetiology. Indeed, as recently reviewed (Dranoff and Wells, 2010), the critical point is likely to be represented by the cross-talk between damaged and/or activated BDEC and PFs rather than the specific aetiology. Accordingly, several investigators have shown the existence of a direct correlation between the intensity of the so-called ductular reaction (a peculiar form of hyperplastic response of BDEC) and the severity of fibrosis in human liver disease of a variety of etiologies, including chronic HCV and NASH, as well as in animal models (Beaussier et al., 2007; Clouston et al., 2005; Fabris et al., 2007; Richardson et al., 2007). In particular, IL-6 and MCP-1 are emerging as important mediators of cell–cell communication between BDEC and PFs, with IL-6 being expressed specifically by BDEC and PFs expressing the IL-6 coreceptor glycoprotein 130 (gp130). It has been proposed the existence of a paracrine loop in which IL-6, by down-regulating NTPD2 on PFs (without

altering PF myofibroblastic differentiation), can favor proliferation of BDEC that, in turn, provide stimuli like TGFβ2, PDGF and MCP-1 that sustain proliferation and MF-like differentiation of PFs.

As a final comment for the present section on the putative role of PFs and P/MFs in liver fibrogenesis, one should note that as proposed by Dranoff and Wells (2010) PFs and P/MFs may be as multifunctional as activated HSCs or HSC/MFs, then having a role in the liver progenitor cell niche and in hepatic progenitor cell expansion and differentiation (see paragraph 4 dedicated to the roles attributed to hepatic MFs for more details).

3.3 Bone marrow-derived cells as an extra-hepatic source of MFs

Clinical and experimental evidence indicates that under conditions of chronic liver injury, pro-fibrogenic MFs (mainly IF/MFs and, possibly, some P/MFs) may also originate from progressive recruitment of bone marrow-derived cells (Forbes et al., 2004; Kisseleva et al., 2006; Kallis et al., 2007; Henderson and Forbes, 2008; Friedman, 2008b; Parola et al. 2008; Forbes & Parola, 2011; Kisseleva & Brenner, 2011). This extrahepatic source of MFs is at present believed to offer a significant although modest contribution to liver fibrogenesis. Moreover, such a contribution, from a quantitative point of view, is likely to vary depending on both the aetiology and the progression rate of the specific form of CLD.

From an historical point of view, the first evidence for a bone marrow-derived origin of hepatic MFs was provided in 2004 (Forbes et al., 2004) in a study in which were analysed fibrotic liver obtained from two different kind of patients: a) from male patients (affected by CLDs of different aetiology) that had received liver transplants from female donors and subsequently developed CLD; b) from a female patient that received bone marrow transplant from a male donor and afterward developed HCV-related cirrhosis. By employing fluorescence in situ hybridization (FISH) for the Y chromosome together immune-histochemistry for MFs specific antigens, Authors showed unequivocally that a significant numbers of Y chromosome in fibrotic areas of were found in the nuclei of α-SMA positive cells having a MF phenotype. In the liver transplant cases, a percentage of α-SMA positive MFs ranging from 6.8% to 22.2% contained the Y chromosome whereas in the female recipient of a bone marrow transplant from a male donor, 12.4% of the MFs were positive for the Y chromosome. This first study was followed by other carefully designed experimental studies that confirmed the concept (i.e., MFs may originate from circulating cells derived from bone marrow recruited into chronically injured liver) and were instrumental to identify at least two distinct populations of bone marrow-derived MFs precursors, including circulating fibrocytes (Kisseleva et al., 2006) and mesenchymal stem cells (MSC; Russo et al., 2006; Valfrè di Bonzo et al., 2008).

In their study Kisseleva and coworkers performed experiments in which chimeric mice, transplanted with donor bone marrow from collagen alpha1(I)-green fluorescent protein (GFP)+ reporter mice, were subjected to the BDL model for biliary fibrosis. In response to injury, bone marrow-derived collagen-expressing GFP+ cells were detected in the liver tissues of chimeric mice and these bone marrow-derived cells, that were negative for α-SMA and desmin (then not originating from HSC/MFs), were found to co-express collagen-GFP+ and CD45+. This led Authors to suggest that these cells were representing a unique population of circulating fibrocytes that, in addition, increased numerically in bone marrow

and spleen of chimeric mice in response to injury. These fibrocytes when cultured in the presence of TGF-β1 differentiated into α-SMA and desmin positive collagen-producing MFs, confirming then to be potentially able to contribute to liver fibrosis.

In the same year Russo and coworkers (Russo et al., 2006) employed female mice that were submitted to an experimental protocol consisting in: a) lethal irradiation, followed by b) transplant of either whole bone marrow or cell population enriched in MSC from mice male donors to be finally submitted to c) different protocols of fibrosis induction. BM-derived cells were tracked through FISH analysis for the Y chromosome FISH and results obtained indicated unequivocally that the bone marrow contributed significantly to hepatic stellate cell and MFs populations. Moreover, these bone marrow - derived MFs were able to actively synthesize collagen type 1 and originated largely from mesenchymal stem cells.

In a subsequent study (Valfrè di Bonzo et al., 2008), non-obese diabetic - severe combined immuno-deficient (NOD-SCID) mice were sub-lethally irradiated, transplanted with highly purified populations of ex-vivo expanded human MSC and then submitted to a protocol of chronic injury in order to induce fibrosis. When chimeric livers were then analyzed for expression of human transcripts and antigens it was found that a significant number of cells of human origin (identified by expression of HLA class I antigens) exhibited a myofibroblast-like morphology. Moreover, human MSC in their MF-like phenotype were found to respond by proliferation and or migration to PDGF-BB and MCP-1 which are known to be effective on HSC/MFs. This strongly suggests that the pattern of polypeptide mediators known to be generated in CLDs may have a role in the hepatic recruitment/engraftment of MSC.

Along these lines it seems correct to mention also a single study (Higashiyama et al., 2009), perhaps quite controversial (see Kallis and Forbes, 2009), that has questioned the real relevance of the phenomenon, suggesting that the contribution to liver fibrogenesis of MFs from bone marrow-derived cells recruited may be negligible.

3.4 Hepatocytes or cholangiocytes are an unlikely sources of MFs through EMT process

Epithelial to mesenchymal transition (EMT) must be envisaged as a fundamental biological process, paradigmatic of the concept of cell plasticity, that leads epithelial cells to lose their polarization and specialized junctional structures, to undergo cytoskeleton reorganization, and to acquire morphological and functional features of mesenchymal-like cells, including the ability to migrate and to produce and secrete components of the extracellular matrix (Cannito et al., 2010). Although EMT and the related opposite process of MET (mesenchymal to epithelial transition) have been originally described in embryonic development (i.e., where cell migration and tissue remodeling have a primary role in regulating morphogenesis in multicellular organisms), extensive literature data have recently provided evidence suggesting that the EMT process is a more general process that may have a significant role in several pathophysiological conditions, including cancer progression and organ fibrosis (Thiery and Sleeman, 2006; Acloque et al., 2009; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009; Cannito et al., 2010).

Pertinent to this chapter, EMT has been proposed to have a pathogenic role in organ fibrosis and particularly in those conditions in which fibrosis may result from chronic and

uncontrolled activation of wound-healing response, then in conditions where progressive fibrogenesis result in the progressive accumulation of ECM components, derangement of tissue and vascular architecture and eventually organ failure. The involvement of EMT in organ fibrosis was first claimed in experimental studies dedicated to the pathogenesis of kidney fibrosis (Iwano et al., 2002; Yang et al., 2002; Zeisberg and Kalluri, 2004) in which it was shown that a significant number of pro-fibrogenic kidney fibroblasts were positive for FSP1 (fibroblast specific protein 1), an antigen believed to be specific for fibroblast-like cells derived from local EMT. More recently, several studies (reviewed in Choi and Diehl, 2009; Cannito et al., 2010) have proposed that pro-fibrogenic cells may originate in CLDs through EMT involving either cholangiocytes (BDEC) or hepatocytes.

EMT of hepatocytes was suggested quite recently in an experimental study (Zeisberg et al., 2007) describing a progressive appearance in the injured livers of FSP-1 positive cells, although less than 10% of FSP-1+ cells were shown to co-express the typical and widely accepted MFs marker α-SMA. In such a study Authors also performed lineage-tracing experiments using *AlbCre.*R26Rstop*lacZ* double transgenic mice in order to investigate whether hepatocytes undergoing EMT may contribute significantly to fibrosis induced by chronic treatment with the hepatotoxin CCl$_4$. They reported that approx. 15% of hepatic cells were FSP-1 positive at the time of severe fibrosis and that approx. 5% of the hepatic cells were co-expressing either FSP-1 and albumin or FSP-1 and β-gal, then suggestive of EMT. Moreover, these authors also performed experiments showing that bone morphogenetic protein-7 (BMP-7), which is known to antagonize TGFβ1 signalling, significantly inhibited progression of liver fibrosis and almost abolished putative EMT-derived fibroblasts. Similar results, were described by another group that employed a transgenic mouse model of Smad7 over-expression in hepatocytes to counteract CCl$_4$ – induced fibrosis (Dooley et al; 2008). The latter study also reported preliminary morphological evidence for "in vivo" EMT in biopsies from chronic HBV patients in terms of positive hepatocyte nuclear staining for SNAI1 (a specific and EMT-related transcription factor).

Involvement of EMT of cholangiocytes or BDEC has been reported in either experimental and clinical conditions associated with a form of biliary fibrosis. The first report was published in 2006 (Xia et al., 2006) and provided evidence suggesting that BDEC undergoing the process of bile ductular reaction in the rat model of secondary biliary fibrosis due to bile duct ligation were co-expressing α-SMA and cytokeratin 19 (CK-19, a BDEC marker that also stain hepatic progenitor cells or HPCs). The same scenario was confirmed in the same model of BDL (rat and murine) by a series of elegant studies from the group of Anna Mae Diehl (reviewed in Choi and Diehl, 2009), the most relevant (Omenetti et al., 2008a, 2008b, and reference therein) being able to describe an apparently clear cause-effect relationships among EMT of BDEC, appearance of portal MFs and biliary fibrosis as well as the closely related major involvement of Hedgehog signalling pathway. Other studies from the same and other research groups described morphological evidence for EMT of BDEC also in liver biopsies from human patients affected by primary sclerosing cholangitis (PSC, Kirby et al., 2008), primary biliary cirrhosis (PBC, Jung et al., 2007; Robertson et al., 2007; Omenetti et al., 2008b) or biliary atresia (Diaz et al., 2008). Similar evidence has been reported EMT of BDEC in post-transplantation recurrence of PBC (Robertson et al., 2007) and the relevance of Hedgehog and TGFβ1-Smad2/3 signalling was reported also in human patients (Jung et al., 2007, 2008; Omenetti et al., 2008b; Robertson et al., 2007; Rygiel et al., 2008).

Following initial enthusiasm, however, a number of issues have very recently questioned whether EMT may be really involved in CLDs. A first cautionary issue is represented by actual re-evaluation of the specificity of FSP1 as a marker of EMT which comes from carefully performed experiments in kidney. Indeed, some studies have revealed that FSP1 is not at all a marker for fibroblasts but rather for leukocytes and other non-fibroblastic cell types (Le Hir et al., 2005; Lin et al., 2008). In addition, a recent elegant fate tracing study (using Cre/Lox techniques) has clearly shown that although genetically labelled primary proximal epithelial cells exposed in culture to TGFβ underwent apparent EMT becoming MF-like cells, no "in vivo" evidence was detected that epithelial cells may migrate outside of the tubular basement membrane and differentiate into interstitial MFs in a model of kidney fibrosis (Humphreys et al., 2010).

Whether liver fibrogenesis is concerned, the group of Brenner has very recently published three elegant experimental studies that are challenging the involvement of EMT of either hepatocytes or cholangiocytes (or BDEC) as a pathogenic mechanism in liver fibrogenesis. In a first study Authors employed triple transgenic mice expressing ROSA26 stop beta-galactosidase (beta-gal), albumin Cre, and collagen alpha1(I) green fluorescent protein (GFP), in order to have hepatocyte-derived cells permanently labeled by beta-gal and type I collagen-expressing cells labeled by GFP (Taura et al., 2010). These engineered hepatocytes underwent changes towards a fibroblast morphology if cultured in the presence of TGFβ1 but when authors isolated hepatic cells from the liver of triple transgenic mice after induction of fibrosis (carbon tetrachloride chronic model) they could not find cells expressing double-positivity for GFP and beta-gal. All beta-gal-positive cells exhibited the typical morphology of hepatocytes and did not expressed mesenchymal markers like α-SMA, FSP-1, desmin, or vimentin. On the other hand, GFP-positive areas in fibrotic livers were coincident with fibrotic septa but never overlapped with X-gal-positive areas and then Authors concluded that type I collagen-producing cells were not originating from hepatocytes.

A very similar conclusion was reached in a second study (Scholten et al., 2010) in which EMT was again investigated with Cre/LoxP system in order to map cell fate CK-19 positive BDEC in CK-19(YFP) or FSP-1(YFP) mice that were generated by crossing tamoxifen-inducible CK-19(CreERT) mice or FSP-1(Cre) mice with Rosa26(f/f-YFP) mice. MET of GFAP(+) HSCs was studied in GFAP(GFP) mice. Transgenic mice were then subjected to bile duct ligation or chronic carbon tetrachloride treatment. When the livers of fibrotic transgenic mice were analyzed specific immunostaining of CK-19(YFP) cholangiocytes showed no expression of EMT markers such as α-SMA, desmin, or FSP-1. Moreover, cells genetically labeled by FSP-1(YFP) expression did not coexpress neither the cholangiocyte marker CK-19 nor E-cadherin. These results led again Authors to conclude that EMT of BDEC were not contributing to liver fibrogenesis in murine models.

The third study by the group of Brenner was even more relevant because provided compelling evidence that FSP-1 (the putative marker of EMT-derived fibroblasts) in either human and experimental CLDs was not expressed by HSC or type I collagen-producing fibroblasts (Osterreicher et al. 2011). Moreover, FSP1-positive cells did not express classical myofibroblast markers, including α-SMA and desmin, and were not myofibroblast precursors in injured livers as evaluated by genetic lineage tracing experiments. According to what already described by studies on kidney fibrosis FSP1-positive cells expressed F4/80

and other markers of the myeloid-monocytic lineage and the overall characterization pointed out that FSP1 was expressed by a specific subset of inflammatory macrophages in liver injury, fibrosis, and cancer that differed from Kupffer cells for reduced expression of MMP-3 and TIMP-3.

Few months ago a study from the laboratory of Rebecca Wells provided what looks like as an unequivocal proof against EMT in the liver as a source of MFs (Chu et al. 2011). This study uses lineage tracing generated by crossing the alpha-fetoprotein (AFP) Cre mouse with the ROSA26YFP stop mouse in order to trace the fate of any cell ever expressing AFP. As expected, all the cholangiocytes and all the hepatocytes were genetically labeled, because they are derived from AFP-expressing precursor cells. Furthermore, AFP+ progenitor cells were also irreversibly genetically marked. The critical result was that after inducing liver fibrosis using different models, none of the resulting myofibroblasts was found to originate from the genetically marked epithelial (AFP+) cells.

As a final comment for this section, at present the real involvement of EMT as a pathogenic mechanism contributing to liver fibrogenesis in CLDs is then more than controversial, with accumulating evidence deposing against EMT from either hepatocytes or cholangiocytes. This has generated an intense debate that the interested reader may find recapitulated in three recently published editorials (Wells, 2010; Popov and Schuppan, 2010; Kisseleva & Brenner, 2011).

4. The role of hepatic myofibroblasts in liver fibrogenesis and chronic liver disease progression

In the previous section we outlined that hepatic MFs may originate from intra- and, to a less extent, extra-hepatic cellular sources; whether the origin of MFs from HSC or PFs is concerned (Friedman, 2008a; 2008b; Dranoff & Wells, 2010), this is likely to occur through a process of activation/transdifferentiation that is believed to involve common mediators, mechanisms and signalling pathways. Although this scenario may apply also to circulating bone marrow-derived cells recruited in the chronically injured liver, at present most of our knowledge derives from "in vivo" and "in vitro" studies performed on activated human or rodent HSC and then in the following sections we will mainly refer to the paradigm of HSC activation and transdifferentiation to HSC/MFs as well as to all those phenotypic responses which have been attributed to HSC/MFs.

As nicely outlined by Scott Friedman and coworkers, under condition of persisting chronic liver injury HSC activation is believed to progress in sequential stages of initiation and perpetuation (Friedman, 2008a; 2008b; Lee & Friedman, 2011). HSC initiation should be envisaged as an early response which is stimulated by several paracrine signals that lead quiescent HSC to acquire a transient and potentially reversible contractile and profibrogenic phenotype. This "transient" phenotype is typically characterized by the rapid induction of platelet-derived growth factor (PDGF)β receptor expression being then primed to respond to several additional growth factors and mediators that, in turn, will be crucial in eliciting major phenotypic responses operated by fully activated MF-like phenotype (i.e., perpetuation). These responses include proliferation, migration/chemotaxis, contractility, excess deposition and altered remodelling of ECM as well as the emerging role in modulating angiogenesis and the immune respose.

4.1 Proliferation and migration/chemotaxis of HSC/MFs

Cultured HSC/MFs proliferate in response to a number of mitogenic stimuli, including primarily PDGF, basic fibroblast growth factors (bFGF), angiotensin II (AT-II), vascular endothelial growth factor A (VEGF-A) and thrombin (Pinzani and Marra, 2001; Friedman, 2008b; Parola et al., 2008). The most powerful mitogenic stimulus is by far represented by PDGF, particularly by PDGF-BB homodimeric isoform which can be released in the scenario of a CLD by either activated Kupffer cells, sinusoidal endothelial cells, platelets or by activated MFs in a well established autocrine/paracrine loop. PDGF-BB mitogenic signalling pathways requires first interaction with PDGF β-receptor subunit (PDGFβR), which is a tyrosine kinase receptor, leading then to the activation of the classic downstream signalling involving Ras/ERK pathway, phosphatidyl-inositol 3-kinase (PI-3K), ERK5 and others (Pinzani and Marra, 2001; Friedman, 2008b). This pathway is also responsible for migratory response since PDGF-BB also represents the best characterized and most potent chemoattractant for HSC/MFs (Pinzani and Marra, 2001) as well as for MF-like cells from human MSC (Valfrè di Bonzo et al., 2008). Indeed, migration/chemotaxis of HSC/MFs and, more generally, of hepatic MFs represents a relevant pro-fibrogenic response that allow MFs to reach the site of injury and to align with both nascent and established fibrotic septa. Migration/chemotaxis of hepatic MFs is also elicited by MCP-1, AT-II, VEGF-A, Angiopoietin-1, C-X-C chemokine receptor type 3 (CXCR3) ligands and, interestingly, ROS (Novo et al., 2007; Friedman, 2008; Novo and Parola, 2008; Parola et al., 2008). Along these lines, a recently published study from our laboratory has established that all chemoattractant polypeptides activate, in both HSC/MFs or MFs derived from MSC, a common signalling involving phosphorylation of ERK1/2 and c-Jun-NH2 kinase 1/2 (JNK1/2) through a redox-dependent mechanism that requires a NADPH-oxidase - mediated increase in intracellular generation of ROS. Moreover, the same pro-migratory pathways can also be elicited by intracellular ROS in a polypeptide-independent manner (Novo et al., 2011).

4.2 ECM synthesis/remodelling by HSC/MFs and the concept of fibrosis reversion

Hepatic MFs are of course the main responsible for excess deposition of ECM components, particularly of fibrillar matrix (mainly collagen type I and III), which represent an undisputed hallmark of fibrotic and cirrhotic livers. Indeed, progressive fibrogenesis is typically characterized by the replacement of the low-density basement membrane of the subendothelial space of Disse with fibril-forming matrix, a scenario that negatively affect differentiated cell functions (mainly of hepatocytes). This scenario is believed to result primarily from a disequilibrium between excess deposition of fibrillar collagens as well as of other ECM components and a reduced/altered degradation and remodelling of fibrotic ECM. According to current literature HSC/MFs and likely all hepatic MFs have a predominant role in modulating both ECM deposition and remodelling.

Hepatic MFs synthesize ECM components primarily as a response to TGFβ1 which can be released in the scenario of a CLD by either activated inflammatory cells, mostly Kupffer cells or monocyte/macrophages recruited from circulation, as well as by HSC/MFs themselves in a paracrine and autocrine manner. TGFβ1 operates mainly through classic downstream signalling and then involving Smads-2 and -3. Connective tissue growth factor (CTGF) and cannabinoids have been also identified as potent profibrogenic signals for HSC/MFs

(Friedman, 2008b) and the list of putative pro-fibrogenic polypeptides also include vascular endothelial growth factor - A (VEGF-A) (Medina et al., 2004; Parola et al. 2008). To this list one should also add leptin, which is a key adipokine that has been implicated in fibrogenesis in relation to NAFLD development towards NASH (Marra & Bertolani, 2009; Lee & Friedman, 2011). In particular, leptin has been reported to promote HSC fibrogenesis and to enhance tissue inhibitor of metalloprotease type 1 (TIMP-1) expression (see later for the role of TIMP-1 over-expression). Leptin operates through its receptor (ObR) and leads to the stimulation of Janus kinase (Jak)-signal transduction and activates the Jak-signal transduction and activator of transcription (STAT) signalling pathway and, partially, through suppression of peroxisome proliferator-activated receptor-γ (PPARγ), the latter being an anti-fibrogenic nuclear receptor able to reverse HSC activation and to maintain HSC quiescence. The action of leptin is usually counteracted by the circulating levels of adiponectin and indeed adiponectin levels have been reported to decrease in liver fibrosis (Marra & Bertolani, 2009; Lee & Friedman, 2011).

Where ECM remodelling is concerned, according to current literature HSC/MFs mainly express metallo-proteinases (MMPs) able to degrade basement membrane (MMP-2, MMP-9, MMP3 or stromelysin) that are less efficient to degrade fibrillar matrix, with a low expression of MMP-1 (interstitial collagenase). HSC/MFs also overexpress tissue inhibitor of metalloproteinase type 1 (TIMP-1) that, in turn, can inhibit interstitial collagenases and act as anti-apoptotic factor for HSC/MFs. Deposition of fibrillar matrix and formation of fibrotic septa is also favoured by the fact that HSC/MFs and, likely, all hepatic MFs, develop resistance to induction of apoptosis (El-Sharkawy et al., 2005; Novo et al., 2006; Friedman, 2008b; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010). Related to this concept are findings of the last decade suggesting that (Iredale, 2007; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010) liver fibrosis and, possibly, initial stages of cirrhosis are potentially reversible in the presence of effective therapy and/or aetiology eradication. Experimental and clinical studies suggest that regression of histopathology develops as a result of increased apoptosis of HSC/MFs and MFs and is paralled by increased expression of interstitial collagenases by hepatic macrophages. However, it should be noted that based on the absence of any unequivocal clinical finding, most researchers still believe that advanced human cirrhosis (i.e., in the presence of a very significant derangement of vascular architecture) is unlikely to regress (Iredale, 2007; Henderson and Iredale, 2007; Parola et al., 2008; Pinzani, 2009; Povero et al., 2010).

4.3 HSCs as liver specific pericytes and the pro-angiogenic phenotype of HSC/MFs

Angiogenesis can be defined as dynamic, hypoxia-dependent and growth factor – mediated process leading to the formation of new vessels from pre-existing ones. Hepatic angiogenesis is considered as a key component of the wound healing response to liver fibrosis and is essential for liver regeneration but it plays a relevant role also in promoting hepatic carcinogenesis and then the angiogenic process is strictly regulated by several factors (Medina et al., 2004; Fernandez et al., 2009; Valfrè di Bonzo, 2010). In this scenario one should first consider that quiescent HSC have been proposed to act as liver specific pericytes and that under conditions of chronic liver injury HSC/MFs acquire features of smooth muscle cells and the ability to contract in response to vasoactive (Friedman 2008a, 2008b; Parola et al. 2008). HSC/MFs contractility is thought to contribute to both the genesis

of increased portal resistance during early stages of fibrosis as well as, by contributing to angiogenesis, to the late and persistent increase in portal pressure found in the cirrhotic liver which is largely due to the distortion of hepatic angioarchitecture (i.e., a consequence of angiogenesis). HSC, in particular, are located in the perisinusoidal space of Disse and with their perisinusoidal processes can affect pericapillary resistance then contributing to modulate hepatic blood flow through contractility. HSC are sensitive to well known vasoactive mediators with contraction being elicited by Endothelin-1 (ET-1) whereas relaxation is induced by nitric oxide (NO) or carbon monoxide (CO).

Under conditions of chronic liver disease, vascular derangement and excess ECM deposition contribute to create an hypoxic milieu which is a major and physiological stimulus for angiogenesis. Along these lines, in recent years it has become clear that HSC may respond to hypoxia and contribute to angiogenesis, particularly when activated to the myofibroblast - like pro-fibrogenic phenotype. The interested reader may refer to recent more comprehensive reviews for more details (Medina et al., 2004; Fernandez et al., 2009; Valfrè di Bonzo et al., 2009). A number of critical concepts and findings correlating MFs, fibrogenesis and angiogenesis should be outlined.

First, angiogenesis and up-regulation of VEGF expression have been documented in experimental models of acute and chronic liver injury as well as in human fibrotic/cirrhotic liver, including chronic infection by HBV and HCV, and autoimmune diseases such as PBC and PSC. Moreover, in both experimental and clinical conditions angiogenesis and fibrogenesis develop in parallel and strict relationships between hypoxia, angiogenesis, VEGF expression and fibrogenesis have been outlined. Along these lines, VEGF expression is mostly limited to hepatocytes and to HSC/MFs and, possibly, other hepatic myofibroblasts. Other polypeptides have been involved in hepatic angiogenesis as a process associated with the fibrogenic progression process in CLDs, including, in particular, leptin and Hedgehog (Hh) ligands.

Where hepatic MFs are concerned, these cells have also been reported to play a significant pro-angiogenic role. As for recent literature data, HSC/MFs can be considered as a hypoxia – sensitive, cyto- and chemokine-modulated cellular crossroad between necro-inflammation, pathological angiogenesis and fibrogenesis (reviewed in Fernandez et al., 2009; Valfrè di Bonzo et al., 2009). This statement is justified by the fact that HSC and HSC/MFs react to conditions of hypoxia and leptin by up-regulating transcription and synthesis of VEGF, Angiopoietin 1, as well as of their related receptor VEGFR-2 and Tie2. Moreover, HSC/MFs respond to the action of VEGF and Angiopoietin 1 in terms of proliferation, increased deposition of ECM components and increased migration and chemotaxis (Novo et al., 2007) in a redox-dependent way involving activation of Ras/ERK and JNK1/2 signaling pathways (Novo et al., 2011).

According to this scenario, in both human and rat fibrotic/cirrhotic livers (Novo et al., 2007) α-SMA-positive MFs able to express concomitantly VEGF, Ang-1 or the related receptors VEGFR-2 and Tie-2, are found at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa. This distribution is likely to reflect the existence of an early phase of CLD, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by HSC/MFs, and of a later phase occurring in larger and more mature fibrotic septa where the chronic wound healing is less active and fibrogenic

transformation more established. In the late setting pro-angiogenic factors are expressed only by endothelial cells, a scenario that is likely to favour the stabilization of the newly formed vessels.

As a final comment, the reader should note that angiogenesis is at present debated as a potential therapeutic target in the treatment of CLDs since the bulk of available experimental data indicate that anti-angiogenic therapy is effective in preventing progressive fibrogenesis (reviewed in Fernandez et al., 2009; Valfrè di Bonzo et al., 2009). Interestingly, to block angiogenesis also results in a significant inhibition of the development of portal hypertension, porto-systemic collateral vessels and hyperdynamic splanchnic circulation.

4.4 HSC/MFs and their role in the modulation of inflammatory and immune response

Persisting inflammatory response in a CLD is considered as one of the major "driving forces" sustaining fibrogenesis. HSC/MF and, likely, all MFs behave like target cells for inflammatory cytokines and other pro-inflammatory signals, including: a) ROS and other oxidative stress - related mediators like 4-hydroxy-2,3-nonenal or HNE, generated as a consequence of hepatocyte injury and necrosis; b) apoptotic bodies (engulfing and activating); c) bacterial endotoxin or other endogenous activators of Toll Like Receptor 4 (TLR4) of innate immunity displayed by HSC/MFs. On the other hand, HSC/MFs have been unequivocally shown to represent the cell source (even in an autocrine manner) of a number of pro-inflammatory molecules, including TLR ligands, MCP-1 or CCL2 and other chemoattractants and chemokines (Pinzani & Marra, 2001; Bataller & Brenner, 2005; Friedman, 2008b).

As a matter of fact, HSC are both a source of chemokines as well as a cellular target for their action: HSC express several chemokine receptors including CXCR3, CCR5 and CCR7 and have been reported to express at least CCL2, CCL3,CCL5, CXCL1, CXCL8, CXCL9 and CXCL10 ligands (Sahin et al., 2010). Within this panel of chemokines, CCR5 interaction with its ligand (RANTES or CCL5) is induced by NF-kB signalling and can stimulate HSC proliferation and migration. Moreover (reviewed in Lee & Friedman, 2011) CCR1, CCR5 and CXCL4 deficient mice have been reported to be associated with reduced inflammation and fibrosis. On the other hand, CXCL9, through its receptor CXCR3 operates as an anti-fibrogenic mediators.

HSC do not only secrete inflammatory chemokines but, by also interacting with immune cells through expression of adhesion molecules (mainly intercellular- and vascular-cell adhesion molecule - 1, ICAM-1 and VCAM-1, respectively) can modulate hepatic immune response in addition to natural-killer (NK) cells, T-cells, dendritic cells and of course professional phagocytes (Kupffer cells and macrophages recruited from peripheral blood) a scenario that is likely to be relevant for fibrogenesis progression (reviewed in Friedman, 2008a; 2008b; Lee & Friedman, 2011). By expanding the concept previously introduced, HSC/MFs also express TLRs and then can respond to the presence of endotoxin (LPS). TLRs, which are pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs) to discriminate the products of microorganisms from the host, are expressed on Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, HSC and hepatocytes in the liver. TLR signaling can induce potent innate immune responses in these

cell types and this is relevant since the liver is constantly exposed to PAMPs, such as LPS and bacterial DNA through bacterial translocation from intestine, particularly in the settings of alcoholic liver disease (ALD). Recent evidence demonstrates the role of TLRs in the activation of hepatic immune cells and HSC during liver fibrosis. Activated human HSC/MFs express not only TLR4 but also CD14 and MD2, forming the LPS receptor; LPS then lead to activation of nuclear factor kB (NF-kB) and c-Jun amino-terminal kinase (JNK) isoforms resulting in the synthesis and release of chemokines and adhesion molecules. Moreover, crosstalk between TLR4 signaling and TGF-beta signaling in hepatic stellate cells has been reported, suggesting an additional pro-fibrogenic mechanism (Aoyama et al., 2010).

Concerning the immune modulatory action, a number of relevant issues should be outlined for HSC and MFs (the interested reader may refer to excellent comprehensive reviews (Unanue, 2007; Friedman, 2008a; 2008b; Lee & Friedman, 2011): a) HSC can act as professional antigen presenting cells (reviewed in Unanue, 2007), able to stimulate either lymphocyte proliferation or apoptosis; b) HSC can regulate leukocyte behaviour and are affected by specific lymphocyte populations, with CD8 lymphocytes being more pro-fibrogenic towards HSC/MFs than CD4 cells; c) HSC cells can induce locally immunotolerance throughout T cell suppression; d) natural killer (NK) cells seem able to selectively kill HSC/MFs, a scenario which is apparently stimulated by interferon and inhibited by ethanol.

4.5 HSC/MFs and their putative role in liver regeneration and cancer

At first the role of hepatic MFs in liver regeneration has been originally limited to the notion that these cells, particularly HSC/MFs, may contribute to sustain liver regeneration (for example following acute liver injury or partial hepatectomy) by producing growth factors for either mature hepatocytes or oval cells, bipotent progenitors of hepatocytes and cholangiocytes (Pinzani & Marra, 2001, Friedman 2008a, 2008b).

Recently, HSC/MFs have been reported to contribute to the so called "liver stem cell niche" and were shown to express the stem cell marker CD133 (Kordes et al., 2007). This has led Authors to propose that HSC and possibly HSC/MFs may then directly differentiate into stem or precursor cells. This is indeed a fascinating hypothesis in the scenario of liver regeneration that may even (see Friedman 2008b) offer a further possible explanation for the fact that fibrosis is a "near-absolute" requirement for the development of hepatocellular carcinoma (HCC). One should consider the following facts and hypothesis: a) neoplastic cells may be envisaged to derive either from hepatic progenitor cells (HPCs) or adult and DNA-damaged hepatocytes being sustained by paracrine or survival factors released by MFs or directly from HSC/MFs through a process of mesenchymal to epithelial transition into HPCs; the latter hypothesis is highly speculative, but supported by the notion that in HSC/MFs operate hedgehog and Wnt signalling, two pathways that have been implicated in stem cell differentiation and cancer. Another study went further in proposing an even more speculative and fascinating scenario (Yang et al., 2008) in which HSC may represent a type of oval cell thus being potentially able to generate hepatocytes to repopulate injured livers. In this elegant study, since quiescent HSC express glial fibrillary acidic protein (GFAP), mice in which GFAP promoter elements regulated Cre-recombinase were crossed with ROSA-loxP-stop-loxP-green fluorescent protein (GFP) mice to generate GFAP-

Cre/GFP double-transgenic mice. These mice were fed methionine choline-deficient, ethionine-supplemented diets to activate and expand HSC and oval cell populations. GFP-positive progeny of GFAP-expressing precursor cells were then characterized by immune-histochemistry. HSC, when activated by liver injury or culture, downregulated expression of GFAP but remained GFP(+); they became highly proliferative and began to co-express markers of mesenchymal and oval cells. These transitional cells apparently disappeared as GFP-expressing hepatocytes emerged, began to express albumin, and eventually repopulated large areas of the hepatic parenchyma. These findings led Authors to suggest that HSC are a peculiar type of precursor cell able to transit through a mesenchymal phase before to differentiate into hepatocytes during liver regeneration.

5. Major mechanisms sustaining liver fibrogenesis: a major focus on ROS and ethanol

According to current literature the mechanisms able to elicit and sustain liver fibrogenesis may be classified, from a general point of view, in three main groups including a) activation of chronic wound healing reaction, b) oxidative stress and c) altered modulation of epithelial-mesenchymal interactions.

The chronic activation of the wound-healing reaction is the most common and relevant mechanism in hepatic fibrogenesis which is characterized by the following key general features: a) the persistence of hepatocellular/cholangiocellular damage with variable degree of necrosis and apoptosis; b) a complex inflammatory infiltrate including mononuclear cells and cells of the immune system; c) the activation of different types of ECM-producing cells (HSC, portal myofibroblasts, etc.) with marked proliferative, synthetic and contractile features; d) marked changes in the quality and quantity of the hepatic ECM associated with very limited or absent possibilities of remodeling in the presence of a persistent attempt of hepatic regeneration (Pinzani & Rombouts, 2004; Parola et al., 2008).

In previous sections the attention has been already focussed on those main MF-related features that have outlined the role of several growth factors and cytokines involved in the chronic wound healing reaction and affecting the pro-fibrogenic potential of HSC/MF. For these reasons we will not further expand this issue and for more informations on pro-fibrogenic mechanisms related to chronic activation of wound healing the interested reader may refer to a number of comprehensive reviews generated within the last decade (Pinzani and Marra, 2001; Parola and Robino, 2001; Pinzani and Rombouts, 2004; Bataller and Brenner, 2005; El-Sharkawy et al., 2005; Friedman, 2003, 2008a, 2008b; Iredale, 2007; Lee & Friedman, 2011; Kallis et al., 2007; Parola et al., 2008). Moreover, since the topic of EMT has been already extensively discussed in the section dedicated to the origin of hepatic MFs the putative and controversial role of this process will be not further analyzed.

Whether the role of a derangement of epithelial – mesenchymal interactions is concerned, such a mechanism has been proposed as a major mechanism underlying fibrogenesis during the course of several cholangiopathies. Cholangiopathies are a group of progressive disorders representing a major cause of chronic cholestasis in adult and pediatric patients, and share a common scenario that involves coexistence of cholestasis, necrotic or apoptotic loss of cholangiocytes, cholangiocyte proliferation (i.e., ductular proliferation) as well as portal/periportal inflammation and fibrosis. All these conditions may be included in those

leading to the pattern of biliary fibrosis and indeed it is well known that the ductular reaction can be considered as the most critical event: intense proliferation of these epithelial cells is associated with significant changes in the surrounding mesenchymal cells (first portal fibroblasts and then HSC with parenchyma invasion) and ECM. It has long been unclear whether the first event was represented by phenotypic changes in proliferating cholangiocytes or by changes in ECM leading to epithelial cell proliferation. However, an intense cross-talk between mesenchymal and epithelial (i.e.cholangiocytes) cells has been suggested to result in a release of cytokines and pro-inflammatory mediators possibly responsible for the overall mentioned scenario in cholangiopathies. As a matter of fact, cholangiocytes are now considered as active "actors" in pathological conditions by their ability to secrete chemokines like IL-6, tumour necrosis factor (TNF)α, IL-8 and MCP-1, as well as pro-fibrogenic factors (PDGF-BB, ET-1, CTGF, TGFβ2) : all these factors can be produced also by infiltrating immune, inflammatory or mesenchymal cells, and may affect in turn both epithelial cells and their intense cross-talk with mesenchymal cells sustaining the fibrogenic response (reviewed in Pinzani and Rombouts, 2004). The interested reader may refer for more details to a recent excellent review on the role of portal fibroblast and portal MFs and their interactions with activated/damaged cholangiocytes (Dranoff & Wells, 2010).

In this section we will then mostly focus our attention on the general role of oxidative stress and reactive oxygen species, with a specific reference to the case of ALD.

5.1 Oxidative stress and ROS in liver fibrogenesis: synopsis of general concepts and findings

Involvement of oxidative stress has been documented in all human major clinical conditions of CLDs as well as in most experimental models of liver fibrogenesis (Parola & Robino, 2001; Novo & Parola, 2008), but it is likely to represent the predominant pro-fibrogenic mechanism mainly in NAFLD/NASH and ASH (and then ALD). Oxidative stress in CLDs, resulting from increased generation of ROS and other reactive intermediates as well as by decreased efficiency of antioxidant defenses, does not represent simply a potentially toxic consequence of chronic liver injury but actively contributes to excessive tissue remodelling and fibrogenesis. In this section just a few major concepts, strictly limited to fibrogenesis, will be recalled and for more details, particularly on aetiology – dependent mechanisms leading to oxidative stress, the reader may refer to more extensive reviews (Parola and Robino, 2001; Bataller and Brenner, 2005; Novo & Parola, 2008; Bataller et al., 2011).

5.1.1 Oxidative stress and liver injury: redox-dependent injury is specific for hepatocytes

CLDs are characterized, whatever the aetiology, by persisting liver injury and hepatocyte loss and severe oxidative stress can be considered as a major cause for both necrotic and apoptotic cell death of parenchymal cells whether resulting from inflammatory flares (i.e., HCV or HBV infection) through increased ROS generation by leukocytes, ethanol consumption, hepatic iron overload, antioxidant status or other conditions. Some relevant concepts should be recalled (reviewed in Novo & Parola, 2008):

a.	First, severe oxidative stress may lead to both hepatocyte necrosis and apoptosis, with necrosis mainly resulting from irreversible mitochondrial damage and/or inactivation

of executioner caspases; moreover, both necrosis and apoptosis can be found on the same section, in association with the other events of the chronic scenario (inflammation, fibrogenesis, angiogenesis, etc.);

b. the level of ROS to which target cells are exposed may be critical in deciding whether these cells may survive or die, as described for the engagement of death receptors (DR) or TLR by respective ligands and the involvement of the critical kinase RIP (from Receptor Interacting Protein); along these lines, ROS-related sustained activation of JNK isoforms is a well characterized event leading to cell death in several conditions; moreover, in hepatocytes NF-kB inhibition sensitize cells to TNF-induced apoptosis by means of JNK sustained activation;

c. ROS-related mitochondrial damage is a typical example of two way-injury since mitochondria can represent not only a source of ROS (particularly when their integrity is deranged) but also a target for their action in relation to cell death; ROS are critical in mediating cell death of fatty hepatocytes due to excess of free fatty acids (FFAs) in the liver of NAFLD and NASH patients; this may happen in FFAs-related up-regulation of TNF, increased Fas ligand binding to Fas (CD-95) or induction of endoplasmic reticulum (ER)-stress and the so called "unfolded protein response" (UPR) ;

d. ER-stress, and then ROS, have been implicated also in hepatocyte apoptosis in chronic hepatitis C and ALD;

e. not all reactive species are dangerous for a target cells: for example, nitric oxide (NO) and related reactive nitrogen species (RNS) can theoretically promote or prevent apoptotic cell death by interfering with either mitochondrial-dependent or -independent signalling pathways.

f. although ROS and more generally oxidative stress can lead to hepatocyte death, human hepatic MFs have been reported to easily survive to ROS, HNE and other pro-oxidants (Novo et al., 2006) and that this relies on the MFs activation-related specific "survival attitude" involving up-regulation of Bcl2, over-activation of pro-survival pathways, including NF-kB-related ones, and down-regulation of Bax (Elsharkawy et al., 2005; Novo et al., 2006). Hepatic MFs can then survive to conditions of oxidative stress usually operating in CLDs that, rather (see later), are more likely to sustain their pro-inflammatory and pro-fibrogenic responses.

5.1.2 ROS and oxidative stress-related mediators affect inflammatory and pro-fibrogenic action of MFs

ROS and other reactive mediators such as 4-hydroxynonenal (HNE, a major aldehydic end-product of lipid peroxidation) can be generated outside MFs, here considered as potential "target" cells, being released either by activated inflammatory cells or injured hepatocytes. Indeed, oxidative stress, presumably by favouring mitochondrial permeability transition, is able to promote hepatocyte death (necrotic and/or apoptotic). In some of clinically relevant conditions generation of ROS within hepatocytes may represent a consequence of an altered metabolic state (like in NAFLD and NASH) or of ethanol metabolism (ALD, see next section), with ROS being then mainly generated by mitochondrial electron transport chain or through the involvement of selected cytochrome P450 isoforms like CYP2E1 (reviewed in Novo & Parola, 2008).

Mediators of oxidative stress, whatever the source, aetiology or metabolic condition, are involved in the up-regulation or modulation of the expression of pro-inflammatory cytokines

and chemokines by different cells, (including inflammatory cells as well as HSC/MFs or, presumably, MF-like cells, mostly through activation of NF-kB (Elsharkawy et al., 2005; Novo et al., 2008). In addition, the following major concepts may be recalled: a) ROS are involved in the process of phagocytosis, possibly by leading to amplification of the stimulating signal that follows engagement of Fc receptors on the surface of phagocytic cells; b) ROS may have a role in apoptosis – related removal of leukocytes during inflammatory responses; c) HNE as well as other 4-hydroxy-2,3-alkenals (HAKs), have been reported to be able to stimulate leukocyte chemotaxis at very low concentrations; d) ROS and HNE elicit in vivo and in vitro up-regulation of the chemokine MCP-1, then sustaining recruitment/activation of monocytes/macrophages and Kupffer cells as well as chemotaxis of HSC/MFs.

On the other hand, oxidative stress – related mediators released by damaged or activated neighbouring cells can directly affect the behaviour of human HSC/MFs: ROS or the reactive aldehyde HNE have been reported to up regulate expression of critical genes related to fibrogenesis including procollagen type I, MCP-1 and TIMP-1, possibly through activation of a number of critical signal transduction pathways and transcription factors, including activation of JNK, activator protein-1 (AP-1) and, only for ROS, NF-kB (Parola and Robino, 2001; Novo & Parola, 2008). It has also been reported that ROS may positively modulate proliferation of rat HSC/MFs but here a discrepancy exist with data with human cells in which ROS and HNE, unable to stimulate cell growth at low pro-fibrogenic doses, may rather inhibit basal and PDGF – stimulated DNA synthesis or even induce cell death. On the other hand, low levels of extracellular superoxide anion, but not H_2O_2 or HNE, are able to stimulate migration of human HSC/MFs through activation of Ras/Erk and JNK1/2 signalling (reviewed in Novo & Parola, 2008; Novo et al., 2011).

In addition to "profibrogenic" extracellular release by neighbouring cells, ROS generation within human and rat HSC/MFs has been reported to occur in response to several known pro-fibrogenic mediators, including angiotensin II, PDGF and the adipokine leptin. ROS generation here depends on activation by the cited mediators of a non-phagocytic NADPH oxidase (NOX) that has been detected in either human or rat HSC/MFs. ROS generated within HSC/MFs are likely to act also as positive modulators or pro-fibrogenic signalling pathways, as shown by the fact that selective inhibition of NOX usually reduce the phenotypic response. Moreover, the potential relevance of ROS generation by NOX in fibrogenesis has been disclosed by a study in which mice lacking p47phox (i.e., a crucial subunit of NOX) were protected from development of experimental fibrosis (Bataller et al., 2003). Interestingly, intracellular and NADPH oxidase – related generation of ROS has been reported to follow "in vivo" and "in vitro" phagocytosis of apoptotic bodies from damaged hepatocytes by HSC/MFs, resulting in up-regulation of procollagen I expression (Zhan et al., 2006). More recently, as previously mentioned, our group has outlined that intracellular generation of ROS either linked to NADPH-oxidase activation following interactions of polypeptides with their receptors (PDGF, VEGF, MCP-1 and others) or in a ligand-independent manner, is a critical event in mediating migration of both HSC/MFs and bone marrow-derived MSC in their MF-like phenotype (Novo et al., 2011).

5.1.3 ROS, lipid peroxidation and immune response in human patients

Another relevant concept to mention is the fact that oxidative stress may contribute to CLDs progression also by affecting immune response. Experimental studies (alcohol fed rodents)

and clinical data (patients affected by ALD, chronic HCV infection or NAFLD) indicate that oxidative stress is associated with the development of circulating IgG antibodies directed against epitopes derived from protein modified by lipid peroxidation products or against oxidized cardiolipin. Of relevance, titre of these antibodies correlate with disease severity and, as recently proposed for NAFLD patients, may serve as prognostic predictor of progression of NAFLD to advanced fibrosis (Albano et al., 2007 and reference therein). Along these lines, it should be also mentioned that a T cell mediated response towards lipid peroxidation derived antigens has been described in patients with advanced ALD (Stewart et al., 2004). Indeed, the body of evidence indicate that immune response triggered by oxidative stress may have a significant role in the progression of ALD, in the worsening of chronic hepatitis C by alcohol intake and, possibly, even in the progression of NAFLD.

5.2 Oxidative stress and ROS in alcoholic liver disease

As it is well known chronic ethanol consumption can lead to ALD, which encompasses a large spectrum of pathological liver changes, ranging from simple fatty liver with minimal injury to alcoholic steatohepatitis (ASH) and, in more advanced stages, fibrogenic progression to cirrhosis. Progression of ALD is now considered a multifactorial process involving nutritional, environmental and genetic factors, and ethanol consumption also represents one of the major host-related factors able to accelerate progression of fibrosis towards cirrhosis in chronic HCV patients and, possibly, in patients affected by CLDs with a different aetiology (Bataller & Brenner, 2005; Friedman, 2008b; Parola et al., 2008).

In the last two decades the role of ROS and oxidative stress in the pathogenesis of ethanol-induced liver injury has been extensively investigated (Arteel, 2003; Day & Cederbaum, 2006; Albano, 2008; Novo & Parola, 2008; Cubero et al., 2009) and here a number of relevant concepts may be recalled.

5.2.1 Major redox-related features in ALD

A first relevant issue is represented by the fact that both experimental and clinical data indicate unequivocally that oxidative stress and lipid peroxidation are involved, with antioxidants and free radical scavengers being able, at least in animal models, to afford prevention (reviewed in Novo & Parola, 2008; Cubero et al., 2009). Moreover, ALD progression is accompanied by a progressive decrease in hepatic antioxidant defenses, suggesting then that both increased generation of ROS and decreased ability to inactivate or metabolize ROS have a pathogenic role.

As a second point, one should note that ethanol-related ROS can be produced by the mitochondria respiratory chain, ethanol metabolizing (and ethanol-inducible) cytochrome P450 2E1 (CYP2E1) in hepatocytes, but not in HSCs, and NOXs of activated Kupffer cells or infiltrating neutrophils; moreover, NO produced by NO synthase of Kupffer cells and other reactive nitrogen species (RNS) has also been shown to contribute to ethanol-dependent hepatic injury (Arteel, 2003; Day & Cederbaum, 2006; Albano, 2008; Novo & Parola, 2008; Cubero et al., 2009). Indeed, in addition to the well known action of alcohol-dehydrogenase or ADH in metabolizing ethanol (when ethanol levels are low) CYP2E1 is an ethanol-inducible isoform mainly observed in hepatocytes that plays a role in oxidation in the presence of high ethanol circulating levels and in chronic alcohol consumption. CYP2E1

when metabolizing ethanol leads to the generation of acetaldehyde as well as of the ethanol-derived hydroxyethyl radical and major ROS including superoxide radical, hydrogen peroxide and hydroxyl-radical. Ethanol metabolism also leads to lipid peroxidation and related generation of reactive aldehydic end-products such as HNE and malonyldialdehyde (MDA).

Third, ethanol-induced oxidative stress is likely to contribute to liver steatosis found in alcoholics by causing an impairment in either mitochondrial lipid oxidation or lipoprotein secretions, the latter being related to enhanced degradation of ApoB100 and/or oxidative alteration of lipoprotein glycosilation in Golgi apparatus (Albano, 2008; Novo & Parola, 2008). Finally, CYP-2E1 generated ROS and formation of protein adducts by lipid peroxidation products may also affect proteasomal degradation, an event that has been proposed to lead to cytoplasmic aggregates of cytokeratins 8 and 18 and then the formation of Mallory's bodies (Bardag-Gorce et al., 2006).

5.2.2 Ethanol-mediated and redox-dependent effects on MFs related to paracrine release of ROS from surrounding cells, including HSC

When considering the role of redox-dependent events on the ALD-related fibrogenesis one may envisage a rather simplified scenario in which MFs represent the ideal target cells for ROS and other redox-related mediators released by the different hepatic cell populations operating in the complex microenvironment of ALD. Indeed, it is widely accepted that in early ALD (i.e., ASH) initiation of HSC activation is mainly due to paracrine stimulation by injured epithelial cells as well as inflammatory (macrophages plus neutrophils), endothelial as well as immune cells. Whether chronic ethanol consumption is concerned, a number of cell type - related issues (to be reconnected with previously mentioned concepts and findings) should be outlined (Albano, 2008; Novo & Parola, 2008; Cubero et al., 2009; Bataller et al., 2011):

a. Hepatocytes. Under conditions of chronic ethanol consumption hepatocyte contribution depend on the CYP2E1-dependent generation of ROS and on consequent generation of aldehydic end-products of lipid peroxidation (mainly HNE) as well as on the levels of acetaldehyde (ACA). ROS, ACA and HNE have been all connected with paracrine HSC activation. In addition to the already mentioned ability of ROS and HNE to increase collagen production it should be recalled that also ACA can up-regulate transcription of collagen type I (both COL1A1 and COL1A2) through a TGFβ-dependent mechanism. This is relevant since TGFβ is the most potent pro-fibrogenic cytokine being able not only to sustain HSC activation and excess synthesis of ECM components but also to suppress hepatocyte proliferation and even to induce their apoptosis. It has been also suggested that the ethanol-related altered ratio of NAD+/NADH and NADP+/NADPH may also favor an increased synthesis of angiotensin II which has been reported to be a powerful fibrogenic cytokine (Bataller & Brenner, 2005).

b. Kupffer cells (KC). Chronic ethanol consumption has been reported to impair gut permeability, an event potentially leading to overgrowth of Gram negative bacteria and subsequent translocation of endotoxin or lipopolysaccharide (LPS) from the intestinal lumen into portal circulation. LPS can trigger KC activation and indeed the influx of KC coincide with the appearance of HSC activation markers (α-SMA and

PDGF receptor-β or PDGFRβ). In addition to standard "macrophage-like" functions, one should consider that activation of KC leads to ROS generation through NOX, xanthine-oxidase, mitochondria or even CYP2E1 (detected in these cells), with ROS being then able to enhance HSC activation and collagen type I synthesis (Cubero et al., 2009). Along these lines, KC can also produce NO that has been described to counterbalance effect of ROS, then leading to decreased HSC proliferation, contractility and collagen type I synthesis; unfortunately, NO can also rapidly react with superoxide anion to generate peroxynitrite, a very powerful oxidizing intermediate whose effect on the synthesis of collagen type I and ECM components by HSC are at present unknown.

c. Liver sinusoidal endothelial cells (LSEC). Under conditions of chronic ethanol consumption major reactive intermediates like ACA and ROS can induce LSEC injury. Indeed, injured LSEC have been reported to react by up-regulating the expression of fibronectin isoforms and of a number of critical mediators able to target HSC or HSC/MFs including VEGF and leptin (Friedman 2003, 2008b; Marra & Bertolani, 2009). Injured LSEC may also convert latent TGFβ into the active form (Ikejima et al., 2002).

d. HSC/MFs. Once activated, HSC/MFs respond to stress conditions by secreting (paracrine/autocrine loops) inflammatory cytokines and chemokines such as mainly TNFα, TGFβ, IL-6, PDGF and CCL2 as well as other mediators like angiotensin II and the altered panel of MMPs and TIMPs described in section 4.2 . As mentioned before and pertinent to the condition of ALD, exposure to ROS, HNE and ACA HSC/MFs has been reported specifically to up-regulate synthesis of ECM components (reviewed in Novo & Parola, 2008; Cubero et al., 2009).

6. Conclusions and perspectives

The increasing knowledge on the pathogenesis of liver fibrogenesis (the process) leading to hepatic fibrosis (the result) has led to important changes in the clinical interpretation of the relevance of fibrogenic progression of any CLD. At present there is a need for an accurate and effective monitoring of the fibrotic progression of CLDs and for the effectiveness of the currently proposed treatments. Within the last five years more non-invasive/dynamic methods for the evaluation of liver fibrosis have been described that are beginning to be very useful, particularly if associated to the detection of critical non-invasive serum markers. This seems now mandatory in order to overcome the unavoidable limitations (lack of standardization, sampling error, inter-observer variability etc) of liver biopsy, although this invasive procedure is still regarded by most as the "gold standard" for assessing liver histology, disease activity and fibrosis progression. In addition, the identification of the genes involved in the progression of liver fibrosis would hopefully lead to the establishment of prognostic markers indicating a faster progression of fibrogenic CLDs and indeed several ongoing studies are addressing the relevance of gene expression and/or gene polymorphisms in defined subset of patients. However, in order to enable the rational development of therapeutic targets in CLDs we primarily need to continue to increase our knowledge on the complexity of the fibrogenic response, an issue that still remains a major objective of experimental and clinical studies designed to ascertain both the origin of fibrogenic cells (i.e., hepatic MFs) and, most important, of the mechanisms governing their "pathological behavior" in CLDs that results in the

overall ability to sustain fibrotic progression of the disease towards the end-points of cirrhosis and related complications.

7. Acknowledgements

8. References

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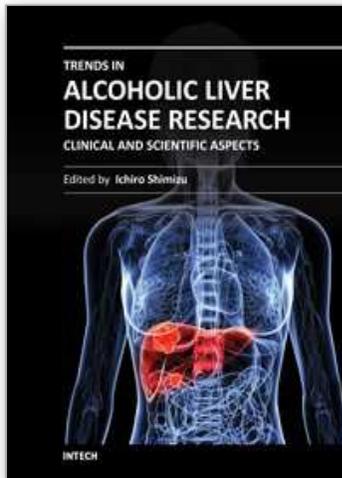
Trends in Alcoholic Liver Disease Research - Clinical and Scientific Aspects

Edited by Dr. Ichiro Shimizu

Alcoholic liver disease occurs after prolonged heavy drinking. Not everyone who drinks alcohol in excess develops serious forms of alcoholic liver disease. It is likely that genetic factors determine this individual susceptibility, and a family history of chronic liver disease may indicate a higher risk. Other factors include being overweight and iron overload. This book presents state-of-the-art information summarizing the current understanding of a range of alcoholic liver diseases. It is hoped that the target readers - hepatologists, clinicians, researchers and academicians - will be afforded new ideas and exposed to subjects well beyond their own scientific disciplines. Additionally, students and those who wish to increase their knowledge will find this book a valuable source of information.

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