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Abbreviations : α-SMA, alpha-smooth muscle actin; BDL, bile duct ligation; COX-2, cyclooxygenase-2; HSC, hepatic stellate cell; HSC-MF, hepatic stellate cell-derived myofibroblast; MCD, methionine-choline-deficient; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; PDGF-BB, platelet-derived growth factor-BB; PDGFR-β, platelet-derived growth factor receptor-beta; PlGF, placental growth factor; PMF, portal myofibroblast; TGF-β, transforming growth factor-beta ; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Abstract

In all fibrotic tissues, the extracellular matrix is produced by myofibroblasts usually characterized by *de novo* expression of alpha-smooth muscle actin (α-SMA). While in the liver, myofibroblasts mainly derive from hepatic stellate cells (HSCs), cells that are distinct from HSCs and located in the portal tract, can also give rise to myofibroblasts that we globally refer to as portal myofibroblasts (PMFs). By different mechanisms that include the formation of direct intercellular junctions with endothelial cells and the production of VEGF-A-containing microparticles, PMFs promote angiogenesis which progresses in parallel with

fibrosis in response to inflammation and fibrosis, in the liver. Hepatic angiogenesis and fibrosis share many signaling pathways, which can be targeted by common therapies. Compared to HSC-MFs, PMFs overexpress COL15A1, which both stabilizes newly formed vessels and forms a scaffold for the deposition of interstitial collagen that accumulates in the fibrotic liver. Thereby, PMFs may provide a critical link between angiogenesis and fibrosis in livers diseases.

The incidence of chronic liver diseases keeps growing worldwide, largely due to the epidemic of the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD). To a great extent, the prognosis of chronic liver diseases is determined by the development of liver fibrosis. However, no anti-fibrotic drug that would prevent the progression of liver fibrosis towards cirrhosis, is yet available and a better understanding of fibrogenesis in the liver is still needed. Liver fibrogenesis is a dynamic process including quantitative and qualitative changes of the extracellular matrix, of which the most prominent is the deposition of type I collagen. These changes progressively disrupt normal liver architecture and result in cirrhosis formation. In the fibrotic liver, like in all other fibrotic tissues, the extracellular matrix is produced by cells usually characterized by *de novo* expression of alpha-smooth muscle actin (α-SMA), known as myofibroblasts.

Liver myofibroblasts

Myofibroblasts form heterogeneous populations of cells, with different possible origins. Current evidence indicates that in the liver, myofibroblasts mainly derive from hepatic stellate cells (HSCs) (Mederacke, et al., 2013). However, cells that are distinct from HSCs and located in the portal tract, can also give rise to myofibroblasts that we now globally refer to as portal myofibroblasts (PMFs) (Lemoinne, et al., 2015, Lua, et al., 2016). PMFs were first described in the setting of biliary-type liver fibrosis, such as that induced by bile duct ligation (BDL) in rats or mice (Kinnman, et al., 2003). PMFs outnumber hepatic stellate cell-derived myofibroblasts (HSC-MFs) at the onset of biliary-type liver injury (Beaussier, et al., 2007), contributing for more than 70 % to liver myofibroblasts, five days after BDL (Iwaisako, et al., 2014). Simultaneously in sinusoids, HSCs undergo phenotypic changes including the overexpression of desmin and of platelet-derived growth factor receptor-beta (PDGFR-β) as well as increased DNA synthesis, yet without fully converting into myofibroblasts, at this stage (Beaussier, et al., 2007). Studies of PMFs in culture demonstrated that they required transforming growth factor-beta (TGF-β) and that they were also dependent on mechanical

tension, for myofibroblastic differentiation (Li, et al., 2007), a typical feature of myofibroblasts (Eyden, 2008). *In vivo*, the emergence and expansion of PMFs can be triggered by interactions with ductular epithelial cells, which proliferate after bile duct injury in the so-called ductular reaction. Thus, a marked increase in the expression of αvβ6 integrin on the surface of ductular epithelial cells is induced by acute bile duct obstruction in mice, which directly triggers periductal accumulation of myofibroblasts and fibrosis, through the activation of TGF-β (Wang, et al., 2007). In human liver tissues, the expression of αvβ6 in ductular epithelial cells, was also found to be increased in acute, but not chronic, biliary-type injury (Wang, et al., 2007). Reactive ductules also express profibrogenic factors such as TGF-β itself or platelet-derived growth factor-BB (PDGF-BB), which also stimulates PMF expansion (Kinnman, et al., 2003). Yet, the biology of PMFs remains poorly known, mainly due to the lack of markers that would allow investigators to authenticate and distinguish them from HSC-MFs, in the injured liver. In a majority of studies, HSC-MFs have been identified on the unique basis of α-SMA expression, one of the many markers they have in common with PMFs. Therefore, the contribution of PMFs may have been underestimated, and some of their properties improperly allocated to HSC-MFs. By comparing the transcriptome of PMFs and HSC-MFs, we identified a marker of PMFs, virtually not expressed in HSC-MFs nor in any other liver cell type, *i.e.* collagen, type XV, alpha1 (COL15A1) (Lemoinne, et al., 2015), and this result was subsequently confirmed by another group (Lua, et al., J Hepatol 2016). We showed that both in animal models and in patients with chronic liver diseases, a marked increase in the hepatic expression of COL15A1 occurred at the time of progression to advanced liver fibrosis (Lemoinne, et al., 2015). This was the case not only in biliary-type liver fibrosis (*i.e.* bile duct-ligated rats and patients with primary biliary cholangitis) but also in post-necrotic liver fibrosis (*i.e.* carbon tetrachloride-treated rats and patients with NAFLD), suggesting that PMFs or at least sub-populations of PMFs were implicated in the progression of all types of liver fibrosis. Besides α-SMA expression, myofibroblasts are characterized by ultrastructural features, including a prominent rough endoplasmic reticulum, a Golgi apparatus producing collagen secretory granules and peripheral myofilaments (Eyden, 2008). Yet another highly characteristic trait, is a cell-to-matrix junction, consisting of an aligned myofilament bundle and fibronectin fibril contacting one another through a point at the cell surface, termed fibronexus (Eyden, 2008). Of particular interest in this respect, we found in our previous comparative analyses that PMFs compared to HSC-MFs expressed fibronectin at higher levels and virtually no desmin, that both belong to the definition of myofibroblasts (Eyden, 2008). Therefore, PMFs fulfill more criteria that define myofibroblasts than HSC-

MFs do (Eyden, 2008).

Liver angiogenesis

Angiogenesis is a dynamic process leading to the formation of news vessels from preexisting vessels. In all tissues, angiogenesis is determined by two main pathways, *i.e.* hypoxia and inflammation, and comprises the following steps: i) sprouting and budding of endothelial cells; ii) degradation of the extracellular matrix by proteinases and migration of endothelial cells; iii) endothelial cell proliferation, tube formation and branching; iv) vessel maintenance, maturation and stabilization (Elpek, 2015). Vascular endothelial growth factor (VEGF) signaling is implicated in virtually all steps of angiogenesis, while the recruitment of mural pericyte-like cells is required in the last step, for nascent vessels to mature and stabilize. It is now well established that liver disease progression is accompanied by angiogenesis. The first demonstration was provided more than thirty years ago by Rappaport *et al.*, who showed that human cirrhotic livers contained more vessels than healthy livers, and that cirrhotic nodules were surrounded by a dense vascular plexus (Rappaport, et al., 1983). Liver tissue hypoxia occurs at early stages of liver injury and increases with disease progression, as a result of structural and functional changes in the liver angioarchitecture (Corpechot, et al., 2002). During liver fibrogenesis, fibrillar type I collagen progressively replaces type IV collagen in the perisinusoidal space of Disse, which together with the loss of endothelial fenestrations, causes sinusoidal capillarization. Therefore, fibrosis by itself can contribute to the development of hypoxia, and thereby promote angiogenesis. Vascular remodeling leading to capillarization of the sinusoids and generation of intrahepatic shunts characterizes hepatic angiogenesis. Such changes in angioarchitecture cause a decrease in hepatocyte perfusion, that aggravates hypoxia. Liver angiogenesis is also stimulated by inflammation. Chemokine-dependent accumulation of monocyte-derived macrophages is an important mechanism of hepatic inflammation and fibrogenesis, in human liver diseases and experimental mouse models. The chemokine receptor CCR2 and its ligand CCL2 (MCP-1) promote the accumulation of monocyte-derived macrophages releasing pro-inflammatory and pro-angiogenic cytokines in the liver. Infiltrating CCL2-dependent inflammatory monocytes also provide pro-angiogenic signals, via the production of VEGF-A and matrix metalloproteinase (MMP)-9 (Ehling, et al., 2014). Three-dimensional micromorphological analyses in mouse models of carbon tetrachloride- or bile duct ligation-induced liver injury, demonstrated that macrophage-dependent angiogenesis during chronic liver injury was largely confined to portal veins and that pharmacological inhibition of CCL2-mediated inflammatory monocyte

infiltration primarily reduced angiogenic vessel sprouting in the portal vein (Ehling, et al., 2014). Therefore, infiltrating bone marrow-derived inflammatory monocytes would mediate the induction of hepatic angiogenesis by effects that are primarily attributable to changes in the portal vein system.

Angiogenesis and fibrosis progression closely correlate in experimental liver injury and human liver disease. Angiogenesis and fibrogenesis are also triggered by similar pathways in response to hypoxia and inflammation, so that a potential causal relationship between them has been difficult to establish. In fact, dichotomous effects of angiogenesis on fibrosis have now been reported, in different tissues. The administration of VEGF, while stimulating angiogenesis, has been shown to reduce renal fibrosis and to stabilize renal function, in the remnant kidney model of progressive renal failure (Kang, et al., 2001). Proangiogenic activity induced in an adipocyte-specific VEGF-A overexpression model, has shown to suppress fibrosis, inflammation and insuline resistance during the early phase of high fat diet-induced adipose tissue expansion. Conversely, VEGF-A-VEGFR2 blockade had an aggravating effect in this context, whereas in *ob/ob* mice with preexisting adipose tissue dysfunction, the anti-angiogenic action of VEGF-A-VEGFR2 blockade caused an improvement in metabolism and a decrease in inflammatory factors (Sun, et al., 2012). A large number of studies in animal models of liver injury showed that the inhibition of angiogenesis led to a decrease in liver fibrosis (Table 1). However, the pathways targeted in these studies, could have also promoted fibrosis, directly. In addition, a few studies led to different conclusions. Thus, pharmacological inhibition of the vitronectin receptor integrin alphavbeta3 which stimulates endothelial cell proliferation and HSC activation, while suppressing hepatic angiogenesis, aggravated liver fibrosis induced by BDL or thioacetamide (Patsenker, et al., 2009). Pharmacological inhibition of the chemokine CCL2 reduced monocyte infiltration and angiogenesis but not fibrosis progression in mouse models of carbon tetrachloride or BDL-induced liver injury (Ehling, et al., 2014). Dichotomous effects of angiogenesis were also reported in a model of fibrosis resolution generated by cholecystojejunostomy that restored bile flow after BDL (Yang, et al., 2014a). In this model, VEGF-induced angiogenesis promoted fibrogenesis after bile duct ligation but was also required for fibrosis resolution after cholecystojejunostomy. Accordingly, VEGF-neutralizing antibodies prevented the development of fibrosis but also disrupted hepatic tissue repair and fibrosis resolution (Yang, et al., 2014a).

Contribution of portal myofibroblasts to liver angiogenesis

HSC-MFs and PMFs likely both contribute to liver angiogenesis, although at different stages. HSCs in their quiescent state, act as pericytes that regulate the functions of sinusoidal endothelial cells. Following myofibroblastic differentiation, they acquire a proangiogenic phenotype and secrete proangiogenic factors such as angiopoietin-1 (Semela, et al., 2008, Thabut, et al., 2011). At an early stage of liver injury, HSC-MFs promote enhanced coverage of sinusoids and angiogenesis in areas of active fibrogenesis at the leading edge of developing fibrotic septa (Novo, et al., 2007). At later stages, endothelial cell proliferation correlate with the expansion of PMFs, which has suggested a role of PMF in liver angiogenesis (Lemoinne, et al., 2015). Further evidence that this was the case, was provided by the immunostaining of human cirrhotic livers showing that COL15A1-positive PMFs displayed a perivascular distribution and outlined vascular capillaries within large fibrotic septa. Using a cell model of PMFs that we previously described in detail, we demonstrated that PMFs were able to enhance angiogenesis *in vitro* and *in vivo,* by different mechanisms including the formation of direct intercellular junctions with endothelial cells and the production of VEGF-A-containing microparticles (Lemoinne, et al., 2015). Compared to HSC-MFs, PMFs largely overexpress COL15A1 and also COL18A1, which are the α1 chains of collagen XV and collagen XVIII, respectively. Both collagens belong to the superfamily of multiplexins and their C-terminal parts, endostatin and restin, respectively are anti-angiogenic, which could provide a negative retrocontrol in PMF-induced angiogenesis. Collagen XV provides stability and resilience to mechanical forces in the skeletal muscle and microvessels. It is contained in the basement membrane of continuous capillaries, serving as a scaffold that anchors the basement membrane to interstitial collagen, and loss of its expression results in collapsed capillaries in mice. It is absent from specialized capillaries like fenestrated liver sinusoids, and the normal liver contains almost no collagen XV, with the exception of trace amounts in the portal and periportal area. Collagen XV forms a proangiogenic matrix for endothelial cells, and thereby could contribute to the angiogenic properties of PMFs. Increased expression of collagen XV has been reported in hepatocellular carcinogenesis in mice and humans. In human hepatocellular carcinoma, collagen XV was identified as a prominent histopathological component of intratumoral capillaries (Kimura, et al., 2016).

In summary, PMFs appear to be critical in pathological angiogenesis, which constantly occurs in advanced liver fibrosis. While it remains to be elucidated how angiogenesis and fibrosis are linked to each other during the progression of liver diseases, we suggest that COL15A1-

producing PMFs could provide an important link by both stabilizing newly formed vessels and forming a scaffold for the deposition of interstitial collagen (Figure 1).

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Figure legend

Figure 1. Role of portal myofibroblasts in liver angiogenesis and fibrosis.

A) In normal liver, quiescent hepatic stellate cells (HSCs) for a continuum with portal mesenchymal cells, which include vascular smooth muscle cells (SMCs), portal fibroblasts and few mesenchymal progenitor cells. The extracellular matrix is poorly abundant and contains trace amounts of COL15A1 along mesenchymal progenitors. B) In advanced fibrosis, portal myofibroblasts (PMFs) proliferate and promote angiogenesis. COL15A1 expressed by PMFs provides a scaffold for interstitial collagen produced in excess mostly by hepatic stellate cell-derived myofibroblats (HSC-MFs).

Table 1. Effects of angiogenesis inhibition on liver fibrosis in experimental models

Reference	Animal model	Anti-angiogenic treatment	Assay and change in liver fibrosis
(Yoshiji, et al., 2003)	Mouse - CCl_4	Anti-VEGFR-1 Ab Anti-VEGFR-2 Ab	Hydroxyproline (↘)
(Tugues, et al., 2007)	Rat - CCl_4	Sunitinib (multiple TK inhibitor)	Masson trichrome staining (↘)
(Taura, et al., 2008)	Mouse - CCl_4, BDL	Blockade of angiopoietin signaling by AdsTie2	Hydroxyproline, Sirius red staining (↘)
(Mejias, et al., 2009)	Rat - BDL	Sorafenib (multiple TK inhibitor)	Sirius red staining (↘)
(Patsenker, et al., 2009)	Rat - BDL, TAA	Cilenglinide (αvβ3/αvβ5 integrin inhibitor)	Hydroxyproline, Sirius red staining (↗)
(Thabut, et al., 2011)	Rat - BDL	Sorafenib	Magnetic resonance elastography, Sirius red staining (↘)
(Van Steenkiste, et al., 2011)	Mouse - CCl_4	Anti-PlGF Ab	Sirius red staining (↘)
Hennenberg *et al.* 2011 (Hennenberg, et al., 2011)	Rat - BDL	Sorafenib	Sirius red staining (↘)
May *et al.* 2011 (May, et al., 2011)	Mouse	Blockade of VEGF signaling by transgenic conditional induction of a VEGF decoy receptor	Goldner staining (↗)
(Sahin, et al., 2012)	Mouse - CCl_4	CxCl9 (angiostatic chemokine)	Hydroxyproline, Sirius red staining (↘)
(Huang, et al., 2013)	Rat - CCL_4	Bevacizumab (anti-VEGF Ab)	Hydroxyproline, Sirius red staining (↘)
(Gao, et al., 2013)	Rat - TAA	Celecoxib (COX-2 inhibitor)	Masson trichrome staining (↘)
(Yang, et al., 2014b)	Rat - High-fat/MCD	Sorafenib, Brivanib (muliple TK inhibitors)	Hydroxyproline (↘)
(Yang, et al., 2014a)	Mouse - BDL - BDL followed by cholecystojejunostomy	)) Anti-VEGF Ab)	Hydroxyproline, Sirius red staining (↘) (↗)
(Ehling, et al., 2014)	Mouse - CCl_4, BDL	CCl2 pharmacological	Sirius red staining (➔)

		inhibitor	
(Liu, et al., 2015)	Rat - Dimethylnitrosamine	Sorafenib + gadolinium chloride	Hydroxyproline, Sirius red staining (↘)
(Yan, et al., 2015)	Mouse - CCl_4	Anti-CD147 Ab	Hydroxyproline, Sirius red staining (↘)

AdsTie2, adenovirus expressing the extracellular domain of Tie2; PlGF, placental growth factor; COX-2, cyclooxygenase-2; MCD, methionine-choline-deficient.

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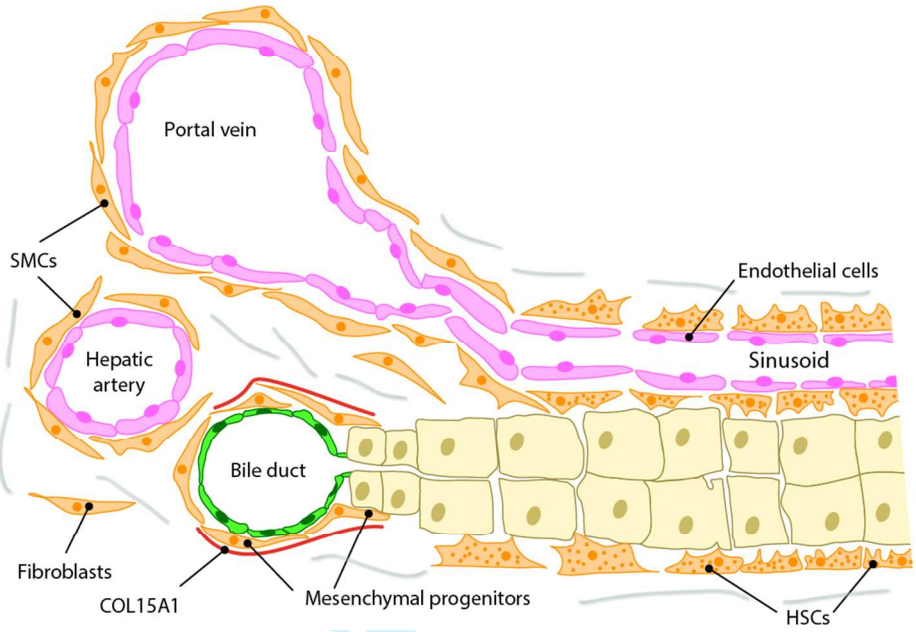
Portal vein
SMCs
Hepatic artery
Bile duct
Fibroblasts
COL15A1
Mesenchymal progenitors
Endothelial cells
Sinusoid
HSCs