can exert their anti-fibrogenic activity by selectively killing early activated HSCs but, remarkably, not quiescent or fully activated and MF-like cells. This phenomenon was first demonstrated by several laboratories in co-culture experiments (i.e., co-culture of HSCs and NK cells), then confirmed in rodent models of CLDs and, even more relevant, in human patients affected by CLD of different etiology [41]. Bing Gao et al. provided evidence for this peculiar and selective killing by showing that early during the process of activation, HSCs produce retinoic acid that, in turn, upregulates the NK cell activating ligand retinoic acid (RA) inducible gene 1 (RAE1) expression on HSCs. RAE1 binds NKG2D on NK cells and subsequently activates NK cells to kill the early activated HSCs through TRAIL- and NKG2D-dependent mechanisms [43,44]. This does not occur in chronically activated HSCs or MFs because these fully activated cells lose cytoplasmic stores of retinol/retinoids and do not produce RA and RAE1, thereby surviving the lethal NK cell attack. This hypothesis is confirmed by studies in NK cells from HCV-infected patients that can induce the apoptosis of activated HSCs through TRAIL-, Fas L-, and NKG2D-dependent mechanisms [45]. The NK cell-induced death of activated HSCs is facilitated by three additional critical features: (i) after activation, HSCs upregulate their expression of TRAIL receptor, which increases their sensitivity to the action of NK cells [46]; (ii) killing of either human and murine HSCs critically depends not only on NKG2D but also on the expression of the NK cell activating receptor NKp46 or of its murine ortholog NCR1, a receptor that recognizes viral hemagglutinins and other unknown ligands [47]; (iii) activated HSCs have been reported to lose the ability to express MHC-1 antigen, an NK cell inhibitory ligand that can suppress NK cell function by binding the inhibitory killer-cell immunoglobulin-like receptors (iKIRs) [48,49]. On the other hand, NK cells have been shown to kill senescent activated HSCs (which accumulate in the fibrotic septa following chronic liver injury) by a different mechanism, involving the granule exocytosis pathway. This represents the consequence of the fact that senescent activated HSCs express elevated levels of NK cell-activating ligands, thus becoming sensitive to NK cell killing [50]. Finally, activated NK cells are known to produce large amount of IFN-γ that has been suggested to contribute to anti-fibrotic effect by directly inducing cell cycle arrest and apoptosis of HSCs [48,51] as well as by enhancing NK cell killing of activated HSCs [43].

The scenario is obviously much more complex and intriguing for the role of NKT cells in relation to HSCs and liver fibrogenesis primarily due to the fact that these cells represent a heterogenous population, with types I and II of NKT cells playing different if not opposite functions in the liver parenchyma [41]. The fact that the mechanism(s) by which NKT cells are activated by endogenous ligands and cytokines is largely unknown adds further variables. Once activated, NKT cells become tolerant and nonresponsive to subsequent stimulation and are able to release high amount of either anti-fibrotic (e.g., IFN-γ) and profibrotic (e.g., IL-4, IL-13,

Hedgehog ligands, and osteopontin) cytokines, together with other cytokines, chemokines, and mediators that can differentially regulate liver fibrogenesis [41]. This is particularly evident by analyzing data from human patients and it has been suggested that this scenario may be significantly affected by the specific etiology. The postulate here is that NKT cells can promote liver fibrogenesis in HCV patients [52] and possibly also in nonalcoholic steatohepatitis (NASH) patients [53]. In the case of chronic viral infections, significant increases in intrahepatic iNKT cells and hepatic expression of CD1d have been reported; in addition, the production of profibrogenic cytokines, IL-4 and IL-13, by iNKT cells was significantly increased in HCV patients with cirrhosis. However, this may not apply to all conditions with NKT cells at times being stimulated to release IFN-γ, perforin or FasL enabling them to induce HSC cell cycle arrest and/or apoptosis, thus potentially inhibiting liver fibrogenesis. According to the hypothesis of Gao and Radaeva, the net effect of NKT cells in progressive CLD is likely regulated by a balance between the anti- and profibrotic effects of NKT cells. Literature data, as reviewed by Gao and Radaeva, also imply that NKT cells may play different roles in the control of liver fibrogenesis in different stages and various types of liver diseases [41].

12.3.3 Cells of the adaptive immunity: Their role in modulating fibrogenesis

As mentioned in the previous section, conventional T lymphocytes are believed to contribute to modulation of liver fibrogenesis in the overall interactions between profibrogenic cells and other cells of innate and adaptive immunity. As for many other tissues and organs, the balance between Th1 and Th2 lymphocytes is likely to affect fibrogenic progression of CLDs also, as indirectly suggested by data from two different strains of mice, with C57BL/6 mice (in which Th1 response predominates) displaying a weaker fibrotic reaction than BALB/c mice (in which Th2 response predominates) [54]. The reference paradigm here is that Th1 cells and of course their cytokines (mainly IL-12 and IFN-γ) may act by limiting/inhibiting fibrogenesis [3,55]. For example, administration of IL-12 has been shown to decrease granuloma formation and fibrosis induced by *Schistosoma* spp. [56]. Evidence in the literature also indicates that IFN-γ can suppress collagen deposition in fibrogenic cells by regulating the balance of MMP and TIMP expression. Moreover, IFN-γ or IL-12 are postulated to also negatively affect the expression and release of profibrogenic cytokines by Th2 cells [57]. In this regard, Th2 cells are believed to exert a predominant profibrogenic action that, apart from the established role in the experimental models by *Schistosoma* spp. [56], mainly relies on the ability of Th2 cells to release IL-13 [3,57]. Indeed, IL-13 availability is critical for at least two reasons: (i) first, this cytokine can stimulate TGF-β1 synthesis and upregulate MMP9 expression, which in turn is known to activate TGF-β1; (ii) second, IL-13 can promote fibrogenesis independently of TGF-β1,

through a mechanism that is controlled by the relative expression of IL-13 receptor-α1 (IL-13Rα1; the signaling receptor) versus IL-13Rα2 (the decoy receptor) on hepatic MFs [57]. The critical role of IL-13 is proven by improvement of liver fibrosis by antagonizing IL-13 signaling [58].

Our understanding of the relevance of the Th1/Th2 paradigm for liver fibrogenesis has been modified by a number of studies outlining the role of Th17 cells as well as regulatory T (Treg) cells. Treg cells, a subset of $CD4^+$ T helper cells, are characterized by the expression of CD25 and by the release of the potent antiinflammatory and immunosuppressive cytokine IL-10. They are able to regulate other immune cells in a dominant-negative manner. The number of Treg cells strongly increase in the liver of patients affected by chronic HCV infection as well as autoimmune liver diseases and primary biliary cirrhosis (PBC) [3]. Data from experimental models suggest that Treg cells possess anti-fibrogenic property. Depletion of Treg cells has been reported to exacerbate fibrosis in the rat BDL model [59], and the anti-fibrogenic function of Treg cells has been attributed to their ability to suppress Th2 cells in *Schistosoma* spp.-induced fibrosis [60] However, it should be noted that $Foxp3^+CD4^+$ Treg cells can release IL-8 in patients affected by chronic HCV infection. Of interest, these IL-8 positive Tregs were found in close proximity of fibrotic areas and their number was found to correlate with the stage of fibrosis *in vivo*. Moreover, in primary human HSCs, these Tregs were found to upregulate the expression of classic genes related to activation, including TGF-β1, α-sma, procollagen, CCL2, TIMP1, and MMP2 in an IL-8-dependent manner [61].

On the other hand, Th17 cells, which are characterized by expression and release of IL-17 and IL-22, have emerged as putative profibrogenic cells and are usually increased in number (serum and liver tissue) in patients affected by several forms of either acute or chronic liver injury. The role of IL-17, in particular, is believed to be relevant because its receptor is expressed by HSCs, cholangiocytes as well as monocytes, and Kupffer cells. HSCs respond to IL-17 by upregulating collagen type I through activation of the STAT3 signaling pathway. More generally, IL-17 upregulates expression of mediators like IL-1β, IL-6, TNF-α and TGF-β in target cells [62].

Here a final mention should be dedicated to cytotoxic $CD8^+$ T cells by recalling data from an elegant adoptive transfer study in which these cells have been shown to be profibrogenic, a property that was negatively affected by IL-10 [63]. Other studies, performed in mice deficient for either $CD4^+$ or $CD8^+$ T cells, did not apparently confirm such a profibrogenic role of these cells [64] and it has been hypothesized that the role of $CD8^+$ T cells may in the end depend on the underlying etiology that drives the fibrotic process [3]. However, one has to recall a very recent study that has shown that fusion of microparticles released by T cells, which circulate in the blood of hepatitis patients, can result in fibrolytic activation of HSCs that leads to the downregulation of pro-collagen α1 and TGF-β1 by these cells [64].

12.4 INTERACTIONS OF HSCs AND HSC/MFs WITH LSECs: FROM LIVER SPECIFIC PERICYTES TO PRO-ANGIOGENIC CELLS

12.4.1 HSCs as liver specific pericytes in normal and pathophysiological conditions

Under physiological conditions, peri-sinusoidal HSCs reside in the space of Disse and are able to interact through their cytosolic processes (hepatocyte-contacting, inter-sinusoidal or inter-hepatocellular, peri-sinusoidal, or sub-endothelial) with hepatocytes as well as LSECs [1–5,65]. In addition to their significant contribution to vitamin A metabolism and deposition/remodeling of the extracellular matrix components of the space of Disse [1–5,65–67] quiescent HSCs from their strategic position are believed to act as liver specific pericytes and regulate sinusoidal blood flow [66,67]. Indeed, under physiological conditions quiescent HSCs in the space of Disse can maintain contact with sinusoidal endothelial cells through their peri-sinusoidal or sub-endothelial processes that run along one or more sinusoids. Moreover, their secondary processes appear to encircle the sinusoids [65,68] and HSCs are also reached by axonal processes of autonomic nerve containing several vasoactive peptides [65]. Although cultured human HSCs were found to contract in response to vasoconstrictors like thrombin, angiotensin-II, and ET-1 and to relax in response to NO and NO donors, prostaglandin E2, somatostatin, and adrenomedullin (reviewed in Refs. [65,67]), morphological and cell culture data were not sufficient to prove that quiescent HSCs may support or modulate sinusoidal diameter. More compelling data in favor of quiescent HSC contraction were produced from *in vivo* microscopy study [69]. On the other hand, overwhelming literature supports the paradigm that liver injury, particularly when chronic, can lead to vascular disorder in which ET-1 is overproduced by HSCs whereas NO release by LSECs is reduced [2,5,65,67,70]. Along these lines, although ET-1 was originally identified as a potent vasoconstrictor produced mainly by endothelial cells [71], HSCs were recognized by different laboratories as a major source of ET-1 as well as a target for this vasoactive molecule during liver injury [72–75]. ET-1 has a prominent contractile effect on HSCs and MFs, which has been proposed to contribute to portal hypertension in the cirrhotic liver [2,5,65,67,70,72–75] (see Chapter 8).

In a recent review by Iwakiri et al. [70], interactions between LSECs and activated HSCs are believed to be critical in intrahepatic vascular pathophysiology. A relevant point is represented by the regulation of the LSEC phenotype, which is the net result of the action of soluble factors (mainly VEGF, angiopoietins, ephrins, and fibroblast growth factors (FGFs)) as well as mechanical forces like those due to shear stress that, in turn, modulate endothelial NO synthase (eNOS) activity in LSECs, thereby regulating flow and vascular tone in the sinusoids [76]. Mediators released by LSECs in a paracrine manner are also involved in maintaining the "physiological" LSEC

phenotype and can affect the early response of HSCs. In this regard, in response to injury, LSECs produce the cellular isoform of fibronectin that can stimulate in a paracrine way early HSC activation and, more specifically, HSC synthesis of ET-1 [77]. ET-1, in turn, can promote the proliferation of early-cultured HSCs, but inhibits fully activated HSCs (i.e., cultured for more than 1 week) [78]. As mentioned, the LSECs phenotype changes dramatically during chronic liver injury undergoing the so-called "capillarization" in association with a reduction in eNOS activity and NO synthesis after injury, likely due to extensive post-translational dysregulation of eNOS [70]. A reduction in NO release by LSECs may play a role in the fibrogenic progression of CLDs because NO has been shown to maintain quiescence of HSCs and reduced exposure of HSCs to NO may facilitate their activation [79,80]. The very complex scenario of interactions between LSECs and HSCs is critical in the maintenance/alteration of microcirculation during liver injury because HSCs also produce NO (i.e., a physiological antagonist to ET-1) [81], likely as a consequence of the activity of iNOS in response to pro-inflammatory cytokines or endotoxemia (reviewed in Ref. [70]). The overall message here is that all these changes are deemed to facilitate remodeling and constriction of the sinusoidal vasculature, which in turn can increase hepatic vascular resistance and is believed to represent an early feature of intrahepatic portal hypertension (also see Chapter 8).

12.4.2 HSCs and their proangiogenic role in CLD

During chronic liver injury, the sinusoids undergo significant vascular remodeling with changes in the LSEC phenotype and their interactions with HSCs. The most evident change is represented by capillarization of sinusoids, which is characterized by the loss of fenestrae in LSECs and by abnormal deposition of a basement membrane matrix in the space of Disse. In addition to the previously mentioned changes in NO and ET-1 synthesis, a number of paracrine and autocrine interactions have been described to occur between LSECs and HSCs. If one refers to polypeptide mediators produced and released by activated LSECs, it is worth mentioning that they produce PDGF, the most potent mitogenic and chemotactic stimulus for HSCs (both quiescent and activated) and TGF-β1, although the release of the latter cytokine by LSECs is likely less relevant for the progression of a CLD than that released by activated macrophages or by activated HSCs in a paracrine/autocrine loop [1–9,70]. In this scenario, one has to recall that VEGF also has a role in modulating LSEC phenotype. Indeed, under physiological conditions VEGF is the most critical molecule to modulate the size and number of LSEC fenestrae [82–85]. This has been shown *in vitro* by disappearance/restoration of fenestrae upon removing the supply of VEGF [83] as well as *in vivo* by disruption of VEGF signaling in mice carrying conditional deletion for VEGFR1 [85]. In conditions of chronic liver injury interactions between LSECs and HSCs become relevant in relation to the

emerging evidence proposing a close link among hypoxia, angiogenesis, and fibrogenesis in the progression of CLDs [8,70,86]. Hypoxic areas within the liver parenchyma are common stimulus in the development of CLD and hypoxia inducible factors or HIFs have been shown to switch on the transcription of proangiogenic genes [78,79]. The involvement of angiogenesis in the progression of CLDs, is intrinsically related to chronic activation of wound healing and histopathological changes in the liver tissue, with increased deposition of ECMs and formation of fibrotic septa (paralleled by vascular changes) in turn leading to an impairment of oxygen diffusion [8,70,86]. In fact, both sprouting and intussusceptive angiogenesis are likely relevant in normal liver physiology and, particularly, in pathophysiologic conditions (i.e., CLDs but also tumor angiogenesis), with a role also in the genesis of portal hypertension in both intra- and extrahepatic circulation [70]. In this context, data obtained in conditional Notch1 knockout mice have suggested that Notch1 in LSECs may be required for retaining/maintaining fenestration of LSECs and that the loss of Notch1 can result in pathological intussusceptive angiogenesis and the development of nodular regenerative hyperplasia and portal hypertension in intrahepatic circulation [44]. In any case, histopathological analyses of cirrhotic livers indicate an increased number of new vessels in the fibrotic septa and surrounding regenerative nodules [87].

Evidence linking hypoxia and angiogenesis to liver fibrogenesis is now overwhelming based on the fact that angiogenesis and fibrogenesis develop in parallel in human patients and experimental model of CLD. More importantly, experimental antiangiogenic therapy was found to be effective in reducing fibrogenic progression, inflammatory infiltrate, the number of α-sma positive MFs as well as the increase in portal pressure [8,13,70,86–89]. In any event, what is relevant for this section is that VEGF expression, detected in hypoxic areas of chronically injured liver and in proximity of fibrotic septa, is mostly limited, in addition to LSECs, to hepatocytes as well as to activated HSC/MFs [8,12,70,86–88]. HSC/MFs or MFs are likely to be then affected in their behavior by proangiogenic cytokines released by hypoxic hepatocytes and LSECs but at the same time these profibrogenic cells are, in response to hypoxia, an additional source of VEGF-A and angiopoietin 1 and also express related receptors, such as VEGFR2 and Tie-2, in CLDs [12,88,90]. Literature indicates that hepatic MFs, in particular HSC/MFs, also represent a target for VEGF and angiopoietin I, with VEGF being able to stimulate their proliferation and increased deposition of extracellular matrix as well as increased migration and chemotaxis [13,88], the latter response being also significantly elicited in cells just exposed to hypoxia [13]. Oriented migration of liver MFs in response to either hypoxia or VEGF and other chemoattractants relies on a biphasic mechanism: (i) an early phase that is switched on by ROS released by either mitochondria or through ligand-receptor related activation of NADPH-oxidase, then resulting in redox-dependent activation of Ras/ERK and JNKs; (ii) a late and delayed phase of migration depending on HIF-1α-mediated, ROS-stabilized, upregulation of

VEGF expression, resulting in the subsequent chemotactic action of extracellularly released VEGF [13]. In addition to these *in vitro* data, immunohistochemical analysis for HIFs and hypoxia gene targets (VEGF, VEGFR2, angiopoietin 1, Tie2) performed on human and rodent fibrotic/cirrhotic liver has led to the hypothesis that hypoxia, through a HIF-mediated pathway, may affect both the migration of MF-like cells and their proangiogenic behavior, leading these cells to align with developing septa and then drive both fibrogenesis and angiogenic response [13,86,88].

12.5 INTERACTIONS OF HSCs AND PORTAL FIBROBLASTS WITH CHOLANGIOCYTES

12.5.1 Cholangiocytes

The intrahepatic bile ducts construct a complicated network of conduits composed of the cholangiocytes. Besides their important roles in bile modification and transport, cholangiocytes actively respond to the inflammatory reactions associated with (chronic) liver injury. They are also sensitive to bioactive molecules in bile, including endogenous bile acids, lipids, and nucleotides as well as exogenous microbially derived and xenobiotic molecules that regulate cholangiocyte function and phenotype. Cholangiocytes are known to express multiple TLRs and associated molecular machinery and express receptors for cytokines, such as TNF-α, IL-6, IL-8, growth factors, such as PDGF, VEGF, and TGF-β1, and morphogens, such as Hedgehog (Hh) ligands and Notch. Thus, cholangiocytes affect repair and remodeling of the biliary epithelium [91]. Cholangiocytes are the target of various diseases termed cholangiopathies, including (i) inherited disorders, such as Alagille syndrome and cystic fibrosis, (ii) autoimmune disorders, such as primary sclerosing cholangitis (PSC), PBC, autoimmune cholangitis, and graft-versus-host disease, (iii) infection, such as cholangitis due to bacteria, fungi, parasite, or viruses, (iv) drug-induced injury, and (v) ischemic injury [92].

During organogenesis, cholangiocytes are derived from liver stem/progenitor cells (LPCs), which express cell surface markers including epithelial cell adhesion molecule (EpCAM), CD13, and CD133 in adult liver. Cholangiocytes vary in size, shape, and function along the biliary tree [93]. Large cholangiocytes are located at the level of interlobular and major bile ducts and maintain normal biliary homeostasis by expressing different ion channels and transporters at the basolateral or apical domain, and undergo proliferation in acute injury. On the other hand, small cholangiocytes at smaller bile duct branches, including terminal cholangioles and canals of Hering, behave as LPCs, playing a role in initiation and progression of biliary fibrosis. Ductular reaction initiate the differentiation of resting cholangiocytes to reactive cholangiocytes, which secrete multiple cytokines, chemokines, and growth factors recruiting inflammatory and mesenchymal cells [94].

12.5.2 Portal fibroblasts

Portal fibroblasts (PFs) are the resident fibroblasts of the portal tract, which exist in the mesenchyme surrounding the bile ducts. PFs were first described more than 50 years ago in rat liver and are considered to produce ECMs specifically in biliary fibrosis in addition to HSC-derived MFs [95]. Although PFs as well as HSCs are thought to derive from the septum transversum-derived mesothelial cells, lack of reliable markers to distinguish PFs from HSCs has hampered the definition of the roles of PFs in normal and injured liver. PFs are generally labeled with fibulin 2, elastin, IL-6, cofilin, and the ectonucleotidase NTPDase 2. Recently Mederacke et al. used a mouse model carrying a bacterial artificial chromosome (BAC) with a Cre-driven lecithin-retinol acyltransferase and found that 82–96% of fibrogenic MFs were derived from HSCs in seven models of fibrosis although the possibility that PFs are necessary for, in particular, biliary fibrosis could not be ruled out. In particular, bridging fibrosis expanded from portal and central vein area where PFs and desmin-negative cells exist, respectively [96].

One of the characteristics of PFs, distinct from HSCs-MFs, is their production of elastin. As mentioned earlier, PFs were first defined by their expression of fibulin 2 [97], which is a linker protein occupying the interface between microfibrils and their elastin core. Fibulin 2 sequestrates latent TGF-β binding proteins and might thereby mediate the progression of fibrosis. PFs also produce lysyl oxidase (LOX) and the related protein LOX-like 1, which crosslink elastin. Thus, the function of PFs should be considered with regard to elastin metabolism because elastin fibers may provide mechanical stability to the bile ducts and the portal vasculature [98].

With regard to the interaction of PFs with cholangiocytes, neurotrophin receptor p75-positive progenitors of PFs, and cholangiocytes express the laminin α1 and α5 subunits, respectively, which interact with the β1 integrin subunit to maintain the polarity and lumen of the bile ducts [99]. Jhandier et al. reported an interesting observation; PF-derived MFs fail to express ectonucleotidase NTPDase2 (also known as ecto-ATPase or CD39L1), which metabolizes extracellular nucleotides, resulting in the initiation of cholangiocyte proliferation through P2Y receptor activation in chronic cholangiopathies [100].

12.5.3 Interaction between cholangiocytes and mesenchymal cells in cholangiopathies

In the presence of chronic inflammation, the interactions between reactive cholangiocytes, mesenchymal cells, including HSCs, PFs, and MFs, and the inflammatory infiltrates contribute to the formation of biliary fibrosis [101].

Reactive cholangiocytes are a source of proinflammatory and chemotactic cytokines such as TNF-α, IL-1, IL-6, IL-8, MCP-1, and IFN-γ. Among them, MCP-1 initiates proliferation of PFs, their differentiation to PF-derived MFs and their collagen

mRNA expression. Stromal cell derived factor-1 (SDF-1) is a cytokine with chemoattractive properties for inflammatory cells. SDF-1 is selectively upregulated in cholangiocytes in PBC and PSC and recruits CXCR4, SDF-1 receptor-positive infiltrating T lymphocytes [102].

TGF-β is produced by reactive cholangiocytes and is known to stimulate PF differentiation to PF-derived MFs. Reactive cholangiocytes also produce PDGF and stimulate HSC chemotaxis toward bile ducts and conversion of PFs into portal MF. In addition, in BDL mice, both VEGF and VEGF receptors are upregulated in cholangiocytes and stimulate their proliferation. Reactive cholangiocytes are the main source of connective tissue growth factor (CTGF) in experimental BDL animal models. CTGF induces extensive fibrosis in biliary atresia and desmoplastic reactions in cholangiocarcinoma [103].

Soluble, lipid-modified morphogens interact with patched (Ptc), a membrane-spanning receptor on the surface of Hh-responsive cells. Such signaling by Hh ligands controls tissue construction and remodeling. Hh ligands released by MFs activate Hh signaling in reactive cholangiocytes. In PBC patients, Hh ligands and Hh target genes are present in bile ductules and stromal cells. Hh may promote the acquisition of features of epithelial–mesenchymal transition (EMT) by reactive cholangiocytes. It has been reported that Wnt/β-catenin pathways and Notch signaling pathways are also involved in the proliferation and differentiation of cholangiocytes and in the formation of cholangiopathies [104].

12.5.4 Involvement of EMT in biliary fibrosis?

EMT is a phenomenon in which epithelial cells acquire structural and functional characteristics of mesenchymal cells, in particular, MFs [8]. Cholangiocytes are considered to participate in the generation of fibrosis around bile ducts through EMT. In the course of activation, cholangiocytes lose the expression of E-cadherin, CK-7, or CK-19 whereas they express fibroblast specific protein-1 (FSP-1) or vimentin and generate ECMs including collagen, fibronectin, elastin, and tenascin [105]. Cholangiocytes in culture undergo EMT and exhibit a MF-like morphology and express α-sma and collagen I. Harada et al. demonstrated that poly (I:C) increased the expression of basic FGF, S100A4, and Snail and decrease in the expression of epithelial marker, such as CK19 and E-cadherin, and bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI), a TGF-β1 pseudoreceptor, in cultured human biliary epithelial cells indicating the occurrence of EMT [106]. In human liver with PBC, EMT of cholangiocytes was demonstrated immunohistochemically by co-localization of CK-19 and vimentin and the increase in the expression of snail and FSP-1.

However, two recent reports showed, by using genetic labeling technology, no evidence of EMT in case of liver fibrosis in mice. Scholten et al. studied EMT with

Cre-loxP system to map cell fate of K19 cholangiocytes in $K19^{YFP}$ or FSP-1^{YFP} mice under BDL or CCl_4 administration. They revealed that $K19^{YFP}$ cholangiocytes failed to express EMT markers, such as α-sma, desmin, or FSP-1 while FSP-1^{YFP}-positive cells had no expression of cholangiocyte marker K19 or E-cadherin [107]. Chu et al. demonstrated by using Alfp-Cre × Rosa26-YFP mice that, although primary cholangiocytes isolated from the mice underwent EMT in culture under the stimulation with TGF-β1 and TNF-α, no occurrence of EMT was detected in fibrotic liver induced by CCl_4 administration for 3 weeks or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 2–3 weeks, which was determined by co-localization of YFP with S100A4, vimentin, α-sma, or pro-collagen 1α2 [108]. Taken together, these two reports denied the occurrence of EMT in case of mouse models of liver fibrosis. However, surprisingly, more recent reports by Michelotti et al. showed the ability of HSC's differentiation into hepatocytes and ductular cells by using α-sma-Cre-ER^{T2} or GFAP-Cre-ERTM mice crossed with Rosa-Stop-flox-YFP mice [109]. On the contrary, Lua et al. demonstrated by using Mesoderm posterior 1 $(MesP1)^{Cre}$ and $Rosa26lacZ^{flox}$ mice that mesodermal liver mesenchymal cells, including HSCs and PFs, undergo transformation into MFs, but not hepatocytes, cholangiocytes, and oval cells [110]. There has been no fixed scenario to finalize the EMT and mesenchymal–epithelial transition (MET) battle in liver fibrosis. Detailed analyses of the occurrence of EMT in human liver are also anticipated.

12.6 CONCLUDING REMARKS

Quiescent HSCs as well as hepatic MFs play a central role in modulating/regulating either physiological and metabolic responses in normal liver as well as the most critical events (i.e., inflammatory response, innate and adaptive immunity, fibrogenesis, and angiogenesis) that are determinant for the progression of a CLD, respectively. Experimental and clinical studies performed in the last two decades have been fundamental in order to progressively outline the fascinating major features of the very complex scenario of close interactions and molecular crosstalk among HSCs and MFs and all the other liver resident or nonresident cells. Our increased knowledge of the intimate mechanisms involved in these interactions and of their consequences, especially in conditions of disease, has emerged as crucial in order to identify novel diagnostic and prognostic biomarkers as well as to design novel and more selective therapeutic strategies to affect CLD progression.

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CHAPTER 13

Hepatic Stellate Cells and Hepatocyte Survival

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13.1 INTRODUCTION

The liver is exposed to a variety of noxious stimuli such as food and environmental-derived antigens and chemicals, and toxins including gram-negative bacterial endotoxin (lipopolysaccharide, LPS) on a regular basis via the portal vein. Toll-like receptors (TLRs) recognize the gut-derived, pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycans, and RNA/DNA; lipoproteins are recognized by TLR1 and TLR2, double-stranded (ds)RNA by TLR3, LPS by TLR4, Flagelin by TLR5, single-stranded RNA by TLR7 and TLR8, and CpG-containing DNA by TLR9. The cell's response to the PAMPs is dependent upon the presence and association of TLRs with their co-receptors and adaptor molecules (e.g., CD14, MD2, and MyD88) [1]. While the gut-derived factors may have a direct effect on hepatocytes, they are generally taken up by the cells of the hepatic innate immune system including the resident macrophages Kupffer cells and eliminated effectively. Kupffer cells phagocytose (or endocytose) and degrade particulate or soluble matter including microbes and viruses and products thereof such as LPS. During this process, Kupffer cells produce cytokines such as tumor necrosis factor-α (TNFα), interleukin-6 (IL6) and IL1β, and reactive oxygen species (ROS) that kill the invading microorganisms [2–5]. However, the same cytokines and free radicals can elicit damaging effect on hepatocytes. Much of this information has been gathered by the *in vitro* experiments and supported by *in vivo* animal experiments in which Kupffer cells are blocked by chemicals such as gadolinium chloride ($GdCl_3$) or eliminated by liposome-encapsulated clodronate [6]. Over the last decade, increasing evidence has accumulated to indicate that hepatic stellate cells (HSCs) play an important role in hepatic defense or injury mechanism.

HSCs, located in the space of Disse, are in direct physical contact with hepatocytes. Similar to Kupffer cells, HSCs also produce numerous cytokines and chemokines and thus have the ability to regulate hepatocyte survival in physiology and pathology. While HSCs can impart protection to hepatocytes, a recently developed model of HSC

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Deletion of the Collagen-specific Molecular Chaperone Hsp47 Causes Endoplasmic Reticulum Stress-mediated Apoptosis of Hepatic Stellate Cells*

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Background: Although knockdown of heat shock protein 47 (Hsp47) attenuates liver fibrosis, the underlying molecular mechanism is unknown.
Results: Deletion of Hsp47 caused activated hepatic stellate cells (HSCs) to undergo ER stress-mediated apoptosis when autophagy was inhibited.
Conclusion: ER stress-induced apoptosis may underlie the clearance of collagen-producing HSCs.
Significance: Hsp47 could be an attractive therapeutic target for fibrosis treatment.

Chronic liver injury, often caused by alcoholism and viral hepatitis, causes liver fibrosis via the induction of collagen production. In liver fibrosis, hepatic stellate cells (HSCs) are activated and transform into myofibroblasts, which actively produce and secrete collagen into the extracellular matrix. Hsp47 (heat shock protein 47) is a collagen-specific molecular chaperone that is essential for the maturation and secretion of collagen. Here, we used the Cre-LoxP system to disrupt the *Hsp47* gene in isolated HSCs from *Hsp47* floxed mice. Immature type I procollagen accumulated and partially aggregated in Hsp47-KO HSCs. This accumulation was augmented when autophagy was inhibited, which induced expression of the endoplasmic reticulum (ER) stress-inducible proteins BiP (immunoglobulin heavy chain-binding protein) and Grp94 (94-kDa glucose-regulated protein). The inhibition of autophagy in Hsp47-KO HSCs also induced CHOP (CCAAT/enhancer-binding protein homologous protein), which is an ER stress-induced transcription factor responsible for apoptosis. These data suggest that apoptosis is induced through ER stress by procollagen accumulation in Hsp47-KO HSCs when autophagy is inhibited. Thus, Hsp47 could be a promising therapeutic target in liver fibrosis.

Chronic liver injury causes fibrosis and cirrhosis, resulting in organ failure through excess accumulation of extracellular matrix (1). Hepatic stellate cells (HSCs)[3] store vitamin A as lipid droplets and encircle sinusoids in normal liver (2). During liver fibrosis, HSCs differentiate into myofibroblast-like cells in response to inflammatory cytokines such as TGF-β. This process is characterized by the loss of lipid droplets, accelerated production and accumulation of type I collagen, expression of smooth muscle α-actin, and an increased proliferation rate (3). Transient activation of HSCs is thought to be a protective acute response against liver injury, whereas persistent HSCs activation is responsible for fibrosis (1, 3).

Procollagen requires several molecular chaperones and enzymes for it to generate a trimer consisting of three α-chains and to fold correctly in the endoplasmic reticulum (ER). Such molecular chaperones include BiP/Grp78, Grp94 (94-kDa glucose-regulated protein), protein-disulfide isomerase, and Hsp47 (heat shock protein 47) (4–8). The enzymes prolyl 4-hydroxylase, prolyl 3-hydroxylase, and cyclophilin B are required for the stable triple-helix formation of procollagen (9–12). We previously reported that Hsp47 is a collagen-specific molecular chaperone in the ER (13–16). Disruption of the *Hsp47* gene causes embryonic lethality by 11.5 days post coitus because of the disruption of basement membranes and the misformation of collagen fibrils (17–19). When the *Hsp47* gene was disrupted in chondrocytes using the Cre-LoxP system, where *Hsp47* floxed mice were crossed with mice carrying a chondrocyte-specific *Col2α1-Cre* transgene, mice died just before or shortly after birth displaying severe chondrodysplasia and bone deformities with reduced type II collagen production (20). In Hsp47-null fibroblasts, the triple-helix formation and secretion of type

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[3] The abbreviations used are: HSC, hepatic stellate cell; ER, endoplasmic reticulum; MOI, multiplicity of infection; CQ, chloroquine; 3-MA, 3-methyladenine; MEF, mouse embryonic fibroblast; AdControl, control adenovirus; AdCre, adenovirus encoding Cre recombinase.

I collagen are severely perturbed (19, 21). Type I procollagen α-chains that are misfolded and fail to form a trimer in the ER are degraded via ER-associated degradation after retrograde transport from the ER to the cytosol, whereas misfolded α-chains that form a trimer are eliminated through autophagy, a process termed ER phagy (22, 23). In Hsp47-null fibroblasts, type I procollagen is misfolded and forms trimers, which accumulate as detergent-insoluble aggregates in the ER that are eliminated via the autophagic lysosome pathway (22).

Although expression of *Hsp47* is up-regulated by various cytosolic stresses, including heat shock, constitutive expression of *Hsp47* correlates with that of collagen in various organs and collagen-related pathophysiological conditions, including liver fibrosis, connective tissue diseases, and dermal fibrotic diseases (24, 25). A recent study showed that TGF-β, a pro-fibrotic cytokine, regulates Hsp47 production in fibroblast cell lines (26). Fibrotic progression is attenuated by down-regulation of Hsp47 expression (27–31). Because of the pivotal role of collagen-producing cells in fibrosis, clearance of activated HSCs through cell death might be expected to alleviate and recover from liver fibrosis. Therefore, the induction of the death of activated HSCs by suppression of Hsp47 expression may be an attractive therapeutic strategy for liver fibrosis. Actually, suppression of Hsp47 expression was reported to attenuate liver fibrosis via inducing the apoptosis of HSCs (28, 30); however, the reason why apoptosis is induced upon suppression of Hsp47 expression remains unknown. We previously reported that ER stress causes apoptotic cell death with the induction of CHOP expression in *Hsp47*-disrupted mice and cells (18, 20, 22). Accumulation of misfolded proteins in the ER causes ER stress and triggers activation of the unfolded protein response (32). At least three ER transmembrane sensor proteins are activated in the unfolded protein response: PERK (PKR-like endoplasmic reticulum kinase) (33), ATF6 (activating transcription factor 6) (34), and IRE1 (inositol requiring 1) (35, 36). Under ER stress, the unfolded protein response can operate as a pro-survival process through the transient termination of translation, the induction of a series of proteins that coordinately function to renature misfolded proteins, and/or the elimination of misfolded proteins. However, under conditions of persistent ER stress, pro-apoptotic signals are induced through the activation of several transcription factors, such as ATF4 (activating transcription factor 4) and CHOP, downstream of the PERK pathway (37).

In the present study, we investigated the mechanisms underlying the apoptosis of Hsp47-deficient HSCs and the involvement of autophagy in the clearance of misfolded type I procollagen in the absence of Hsp47. We hypothesized that misfolded type I procollagen in the ER might cause ER stress, resulting in the apoptosis of Hsp47-disrupted HSCs when autophagy is inhibited. To this end, we isolated HSCs from the livers of *Hsp47* floxed mice, disrupted the *Hsp47* gene by infecting cells with an adenovirus harboring the *Cre* gene, and performed biochemical and cell biological analyses.

EXPERIMENTAL PROCEDURES

Reagents—Collagenase type I and ascorbic acid were purchased from WAKO (Osaka, Japan). Pronase E was purchased from Roche, DNase I was purchased from Merck, and TGF-β was purchased from PeproTech Inc. (Rocky Hill, NJ). Chloroquine (CQ), 3-methyladenine (3-MA), leupeptin, and pepstatin A were purchased from Sigma Aldrich (St. Louis, MO). Anti-Hsp47 and anti-Grp94 antibodies were purchased from ENZO Life Sciences (Plymouth Meeting, PA), an anti-BiP/Grp78 antibody was purchased from BD Transduction Laboratories, an anti-CHOP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and an anti-GAPDH antibody was purchased from HyTest (Turku, Finland). Antibodies against mouse type I collagen and β-actin were purchased from Millipore Corporation (formerly NYSE: MIL), and those against LC3 and p62 were purchased from MBL (Nagoya, Japan).

Mice—Hsp47 floxed mice were previously generated (20). All experiments using animals were approved by the Kyoto Sangyo University Committee for Animal Care and Welfare.

Isolation and Culture of HSCs—HSCs were isolated from *Hsp47*$^{flox/flox}$ mice as described by Radaeva *et al.* (38). The isolated HSCs were cultured on a plastic dish in low glucose DMEM supplemented with 10% FBS and antibiotic-antimycotic solution (Sigma-Aldrich) and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Adenovirus Infection—Adenoviruses harboring the *Cre recombinase* gene, or the *neo* gene as the control, under the control of the CAG promoter were kindly provided by Dr. Ikeda (Osaka City University, Osaka, Japan) (39). Adenoviruses were purified using the Adeno-X maxi purification kit (Takara Bio Inc., Shiga, Japan), and the titer of the purified virus was determined using the Adeno-X rapid titer kit (Takara Bio Inc., Shiga, Japan). At days 18–31 after their isolation, HSCs were infected with adenovirus at a multiplicity of infection of 17, 20, or 25 and cultured with 136 μg/ml ascorbic acid.

Immunoblot Analysis—HSCs were trypsinized and seeded onto multiwell plate on day 8 after adenovirus infection with 136 μg/ml ascorbic acid and 1 ng/ml TGF-β. At day 4 after seeding, HSCs were treated with or without 20 μM CQ or 10 mM 3-MA for 24 h. HSCs were lysed with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5.0 mM EDTA, 1% (v/v) octylphenoxypolyethoxyethanol, and protease inhibitors (1 μg/ml of leupeptin and pepstatin A) at 4 °C for 20 min. After centrifugation, supernatants and pellets were collected and electrophoresed on a 7, 10, or 15% SDS-PAGE gel (40). Thereafter, the gels were transferred to PVDF membranes with a pore size of 0.45 or 0.22 μm. The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and then stained with specific antibodies. The targeted proteins were detected by a chemiluminescence method using an ECL Western blotting detection reagent or an ECL prime Western blotting detection reagent (GE Healthcare Bio-Sciences) followed by visualization using the LAS-3000 system (Fujifilm, Tokyo, Japan).

RT-PCR and Quantitative Real Time PCR—Total RNA was extracted from HSCs using the RNeasy mini kit (Qiagen), and then first strand cDNA was synthesized using the SuperScript III first strand synthesis SuperMix for RT-PCR and quantitative real time PCR. For RT-PCR, the PCR primers used to amplify mouse Hsp47 were 5′-AAGATGCAGAAGAAGGCTGTCG-3′ (forward) and 5′-CTGTGACACCCCTGAATTTGGT-3′ (reverse), and those used to amplify mouse XBP-1 were 5′-TGAGAACCAGGAGTTAAGAACACGC-3′ (forward) and 5′-TTC-

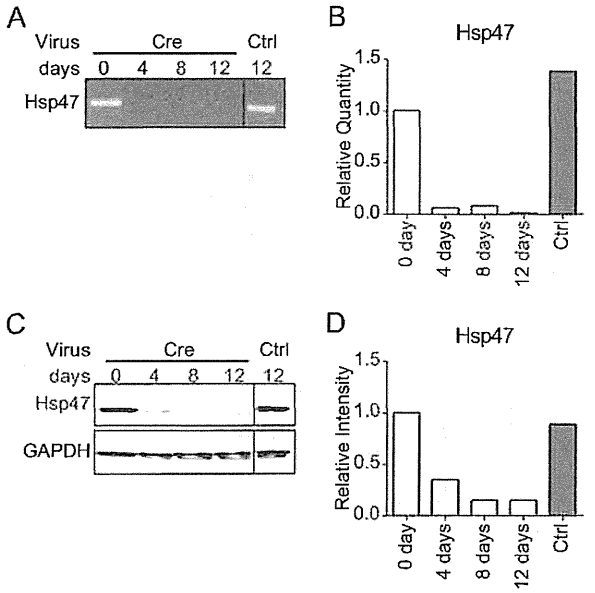

FIGURE 1. **Depletion of Hsp47 in activated HSCs.** *A–C*, the mRNA and protein levels of Hsp47 at the indicated number of days after infection with a control adenovirus (*Ctrl*) or an adenovirus encoding Cre recombinase (*Cre*) at a multiplicity of infection of 17 in activated HSCs were analyzed by semiquantitative RT-PCR (*A*), real time PCR (*B*), and Western blotting (*C*). *D*, the protein level of Hsp47 in *C* is shown relative to that of GAPDH.

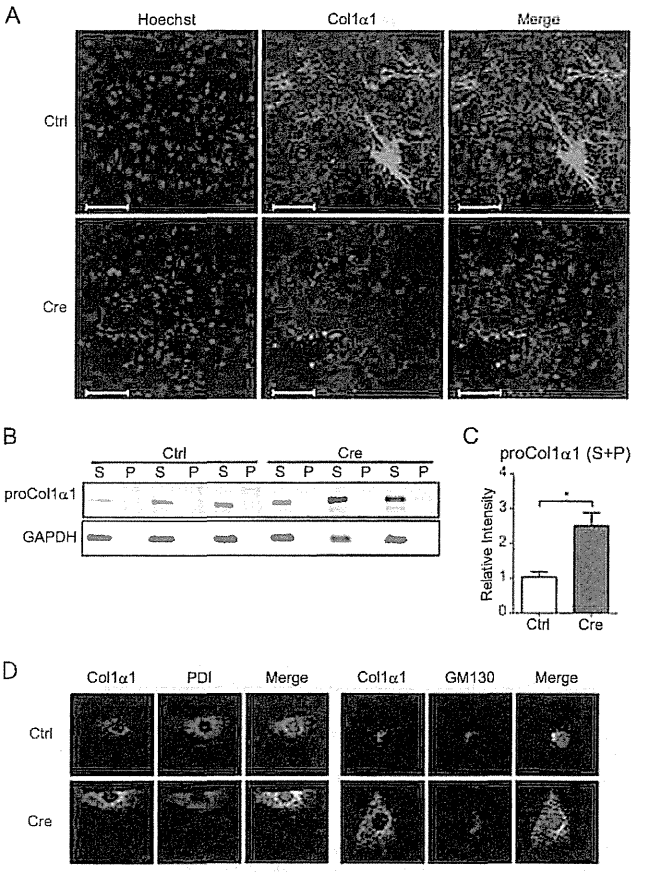

FIGURE 2. **Maturation of type I collagen in Hsp47-KO HSCs.** *A*, deposition of type I collagen in the extracellular matrix after infection of activated HSCs with AdControl or AdCre at a MOI of 17 was detected by staining with an anti-type I collagen (*green*) antibody and Hoechst 33342 (*blue*) without permeabilizing cells. *Scale bars*, 100 μm. *B*, Western blot analyses of type I procollagen α1 in activated HSCs infected with AdControl or AdCre at a MOI of 25. Cell lysates were separated by centrifugation to generate detergent-soluble (*S*) and detergent-insoluble (*P*) fractions. *C*, the protein level of type I procollagen α1 in *B* is shown relative to that of GAPDH. Experiments were performed four times independently, and values are means ± S.E. *, $p \leq 0.05$. *D*, the localization of type I collagen in activated HSCs infected with AdControl or AdCre at a MOI of 20 was determined by staining with anti-type I collagen (Col1α1; *green*), anti-protein-disulfide isomerase (*PDI*; *left*, *red*), and anti-GM130 (*right*, *red*) antibodies and Hoechst 33342 (*blue*). *Ctrl*, control.

TGGGTAGACCTCTGGGAGTTCC-3′ (reverse). For real time PCR, the primers and probes were designed using the Assay Design Center (Roche). The sequences of the primers and the probe used to detect mouse Hsp47 were as follows: 5′-GAAGGCTGTCGCCATCTC-3′ (forward), and 5′-TCCTGCCAGATGTTTCTGC-3′ (reverse), and 5′-TGGTGGAG-3′ (probe). The Universal Probe Library Mouse GAPD Gene Assay (Roche) was used to detect GAPDH as the control. Real time PCR was performed using the Applied Biosystems StepOnePlus real time PCR System (Applied Biosystems, Foster, CA). Expression of Hsp47 was normalized against that of GAPDH.

Immunostaining—HSCs were trypsinized and seeded onto poly-L-lysine-coated cover glasses with ascorbic acid on day 8 after adenovirus infection. At day 4 after seeding, Immunofluorescence was performed as previously described (21). Briefly, HSCs were fixed with 4% paraformaldehyde for 20 min at 37 °C, treated with 0.25% collagenase type I for 3 min at 37 °C, and then treated with 0.1% Triton X-100 for 5 min at room temperature to permeabilize the cells. For staining of extracellular matrix components, HSCs were not treated with collagenase or Triton X-100. HSCs were treated with blocking buffer containing 2% goat serum and 20% glycerol for 30 min at room temperature and then incubated with specific antibodies. Thereafter, HSCs were stained with Alexa Fluor 488-conjugated anti-rabbit IgG or Alexa Fluor 546-conjugated anti-mouse IgG. Immunofluorescence was detected using a LSM-700 microscope (Zeiss, Jena, Germany). The same exposure time was used for the acquisition of all images.

Detection of Caspase-3 Activity—Caspase-3 activity was detected using the NucView 488 caspase-3 assay kit (Biotium, Hayward, CA) according to the manufacturer's instructions. Signals were observed using a BZ-710 microscope (Keyence, Osaka, Japan). The same exposure time was used for the acquisition of all images.

Statistical Analysis—Quantitative data are described as means ± S.E., and the difference between the two groups was statistically analyzed using the two-tailed unpaired Student's *t* test. All quantitative data were obtained from at least three independent experiments. Densitometric analysis of immunoblots was performed using Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan), and the caspase-3 activity signal was calculated using the BZ-X analysis application (Keyence).

RESULTS

Depletion of the Hsp47 Gene in Activated HSCs—To reveal the role of Hsp47 in HSCs, we established Hsp47-KO HSCs using the Cre-loxP system. We previously established cartilage-specific Hsp47-KO mice by crossing *Hsp47*$^{flox/flox}$ mice with mice expressing the *Cre recombinase* gene under the control of