Histol Histopathol (2016) 31: 141-148

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Review

Inducible factors for cancer-associated fibroblasts in liver cancer versus myofibroblasts in inflammatory liver disease

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Summary. The importance of cancer-associated fibroblasts (CAFs) in liver cancer, cholangiocarcinoma (CC) and hepatocellular carcinoma (HCC), has been appreciated in the past 5 years. We focused on how they get activated in the tumor microenvironment in this review. Not only hepatic stellate cells (HSCs) but also portal fibroblasts (PFs) have been appreciated to be key players in liver fibrogenesis, and their different roles have just started to be recognized. Since the role of cholangiocyte in biliary fibrogenic disease might have some similarities to that of CC, we focused on the role of cholangiocytes activating stromal fibroblasts, which would presumably be helpful for better understanding the mechanism of tumor-CAFs interaction. In addition, the activation of CAFs should be different from that of CAFs in HCC, which we consider to be potentially similar to MFs in hepatocyte injury-dependent liver fibrogesis. Herein, we describe the activation of CAFs in CC in comparison to MFs seen in other liver diseases such as 1) MFs in liver fibrosis caused by hepatocyte injury such as alcoholic hepatitis, viral hepatitis, and nonalcoholic steatosis, 2) MFs in liver fibrosis caused by cholestatic disease, and 3) CAFs in hepatocellular carcinoma (HCC). This review on the activation of fibroblasts either in liver cancer or in chronic liver disease would contribute to CAF-targeted therapy in liver cancer.

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DOI: 10.14670/HH-11-668

Key words: Cholangiocarcinoma, Hepatocellular carcinoma, Cancer-associated fibroblast, Hepatic stellate cell, Portal fibroblast

Introduction

Biliary tract cancers are highly aggressive tumors and it is still difficult to achieve early diagnosis or curative treatment (Patel, 2011). They are composed of two major clinical phenotypes; intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC). Although they are more frequently seen in Asia (4-20% of primary liver cancer) than Europe or the United States, the number of patients with ICC is increasing in the United States (Patel, 2001). The most

Abbreviations. ICC: intrahepatic cholangiocarcinoma; ECC: extrahepatic cholangiocarcinoma; HCC: hepatocellular carcinoma; CAFs: cancer associated fibroblasts; TAMs: tumor associated macrophages; TANs: tumor associated neutrophils; HSCs: hepatic stellate cells; PFs: portal fibroblasts; PBC: primary biliary cholangitis; PSC: primary sclerosing cholangitis; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor-β; FGF-2: fibroblast growth factor-2; HGF: hepatocyte growth factor; VEGF: vascular endothelial growth factor; CTGF: connective tissue growth factor; IGF-1: insulin-like growth factor-1; NGF: nerve growth factor; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; MCP-1: monocyte chemotactic protein-1; Hh: Hedgehog; cAMP: cyclic adenosine monophosphate; PKA: cAMP-dependent protein kinase; EGF: epithelial growth factor; NASH, non-alcoholic steatohepatitis; TLR4: Toll-like receptor 4; LPS: lipopolysaccharide; ATX: autotaxin; VASP: Vasodilator-stimulated phosphoprotein; TβRII: TGF-β receptor II; RPS5: ribosomal protein S5.

prominent pathological characteristic in ICC and ECC, which are substantially different from hepatocellular carcinoma (HCC), is a desmoplastic stromal component (Rizvi and Gores, 2013). A major source of this stromal component is cancer associated fibroblasts (CAFs) and collagen produced by them (Terada et al., 1996; Okabe, 2010), although the origin of CAF still remains undefined. In the past five years, the role of CAFs in ICC has been well recognized (Sirica, 2012). Since most previous investigations focused on the factors which are mainly derived from CAFs, this review focuses on how CAFs get activated in liver cancer. In addition, we assumed that the activation of CAFs in cholangiocarcinoma might have some similarities to that of MFs in biliary fibrotic disease but should be different from that of CAFs in HCC. Stromal desmoplasia and collagen deposit are not typical findings for HCC, but an emerging role of CAFs in HCC has been recently appreciated. Therefore, we reviewed 4 types of fibroblasts. Two of them are biliary disease-originated fibroblasts; MFs in biliary fibrotic disease and CAFs in CC. The other two are hepatocyte disease-originated fibroblasts; MFs in hepatocyte-dependent fibrotic disease and CAFs in HCC. A mechanism based classification of activated fibroblasts in the liver revealed their distinct role.

Genetic and pathologic backgrounds of cholangiocarcinoma in relation to CAFs

Genetic and epigenetic characteristics of CC have been largely analyzed in the past 5 years, demonstrating that patients with ICC can be classified based on those molecular characteristics (Andersen et al., 2012; Sia et al., 2013). Famous genetic mutations in CC are *KRAS* (22%), *TP53* (15%), isocitrate dehydrogenase 1 and 2 (*IDH1/2*) (14%), *BRAF* (7%), and *EGFR* (2%) (Tannapfel et al., 2003; Leone et al., 2006; Borger et al., 2012; Chen et al., 2012a; Kipp et al., 2012). Of them, ICC has *IDH1* mutation, whereas ECC predominantly has *KRAS* mutation (Borger et al., 2012). These differences of genetic background in CC presumably affect the feature of stromal component. However, there is no study focusing on the relevance of those mutations to tumor-associated cellular characteristics such as CAFs, tumor associated macrophages (TAMs), and tumor associated neutrophils (TANs). Gene microarray analysis on tumor stromal component demonstrated that genes involved in the activation of hepatic stellate cells (HSCs) were associated with poor prognosis, whereas the percentage of stromal component was not (Andersen et al., 2012). Indeed, we previously showed that abundant stromal fibroblasts in tumor stroma were an indicator of poor prognosis after curative resection for ICC, but the amount of stromal collagen calculated by Sirius Red staining was not associated with poor prognosis (Okabe et al., 2009). Thus, it is certain that CAFs promote CC progression (Sirica et al., 2011; Sirica and Gores, 2014). However it is unclear what converts normal fibroblasts to CAFs and how we can develop the treatment strategy for CAFs.

Another question is how an anatomical location of CC in biliary tree affects the origin of CAFs. HSCs, portal fibroblasts (PFs), and bone marrow derived cells can be candidates for myofibroblasts in ICC (Okabe et al., 2009; Sirica et al., 2011). On the other hand, myofibroblasts in ECC could be predominantly derived from PFs. The difference in origins of CAFs in CC may also depend on the underlying liver disease such as cirrhosis, chronic hepatitis B and C, primary sclerosing cholangitis (PSC), hepatolithiasis, and liver flukes (Palmer and Patel, 2012; Sibulesky et al., 2012). Since a cell specific marker by which we can identify the origin of myofibroblasts is not yet established, this issue should be further addressed in the future.

The origins of MFs in fibrotic liver disease

HSCs have been recognized as the major accelerator in liver fibrosis for several decades (Friedman, 2004, 2008). Once HSCs are cultured on plate, they get activated in a few days. This is presumably the reason why it is difficult to identify crucial factors activating quiescent HSCs into myofibroblasts *in vitro*. Besides, PFs have also been appreciated to play a key role in liver fibrogenesis in the past decade (Kinnman and Housset, 2002; Ramadori and Saile, 2004; Dranoff and Wells, 2010). Major clinical causes of hepatic fibrosis can be divided into two groups based on their cellularity. The former is HSC-associated fibrogenesis caused by hepatocyte injury containing several kinds of hepatitis caused by alcohol, hepatitis virus, and steatosis. The latter is PF-associated fibrogenesis caused by biliary injury and represented by primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). MFs are pathologically typical and essential findings in those fibrotic livers, but the disease-specific mechanism (HSC- or PF- associated fibrogenesis) by which MFs are activated may be of great importance to consider the therapeutic targets. Furthermore, CC and HCC could be defined as PF-assofciated fibrogenic disease and HSC-associated ficrogenic disease, respectively (Fig. 1).

PFs-associated liver fibrogenesis

It has increasingly become clear that MFs derive from not only HSCs but also PFs (Magness et al., 2004). Recent studies have demonstrated that PFs have different phenotype and role in liver fibrogenesis from HSCs (Wells et al., 2004; Li et al., 2007; Bosselut et al., 2010). PFs are considered to play a more important role in cholestatic liver disease than HSCs do, because profibrogenic reactions happen mainly in the connective tissue around portal tracts. Notably, platelet-derived growth factor (PDGF) is the most potent mitogenic stimuli for HSC, whereas it inhibits PFs proliferation and myofibroblastic differentiation. Instead, fibroblast growth factor-2 (FGF-2) induces proliferation of PFs

(Wells et al., 2004; Li et al., 2007). Injured or activated cholangiocytes exert multifunctional capacities based on the specific cholestatic disease, when they are injured and get activated. They can produce 1) growth factors, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), insulin-like growth factor-1 (IGF1), and nerve growth factor (NGF), facilitating adjacent storomal cells as well as parenchymal cells (Cramer et al., 2004; Gigliozzi et al., 2004; Alvaro et al., 2005; Gaudio et al., 2006), 2) cytokines and chemokines, tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL-8, interferon-γ (IFN-γ), and monocyte chemotactic protein-1 (MCP-1), recruiting inflammatory cells (Leon et al., 1997; Cruickshank et al., 1998), and 3) profibrogenic factors, PDGF, TGF-β, and endothelin-1 (ET-1), activating MFs (Gaudio et al., 2006; Luo et al., 2005). Previous *in vitro* studies showed that PDGF-BB increased Shh expression in both cholangiocytes and HSCs, which induced proliferation of HSCs and inhibited apoptosis of HSCs *in vitro* (Omenetti et al., 2008; Yang et al., 2008). PDGF-D is the second most potent isoform in PDGFRβ signaling and promotes the proliferation of MFs induced by bile duct ligation (BDL) in rat (Borkham-Kamphorst et al., 2007). Since both primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are autoimmune disorders

Table 1. Crucial factors inducing PF-associated liver fibrogenesis and CAFs in cholangiocarcinoma.

Factors	Source of cellular type	Disease/material	Species	Ref
Portal fibroblast-associated liver fibrogenesis				
FGF-2	Not shown	Isolated PF	Rat	Li Z, Hepatology 2007
PDGF-D	Not shown	BDL	Rat	Borkham-Kamphorst E, 2007
PDGF-BB, Shh	Cholangiocyte (Shh)	BDL	Rat	Omenetti A, Gut 2008
TGF-β	Not shown	Isolated PF	Rat	Bosselut N, Proteomics 2010
Cholangiocarcinoma				
PDGF-BB	Myofibroblast	CCA model	Rat	Fingas CD, Hepatology 2011
PDGF-D	Cholangiocarcinoma	Xenograft	Hu	Cadamuro M, Hepatology 2013
Shh	Cholangiocarcinoma	Xenograft	Hu	El Khatib, Hepatology 2013
TGF-β1	cholangiocarcinoma	Xenograft	Hu	Claperon A, Hepatology 2013

BDL: bile duct ligation; PF: portal fibroblast

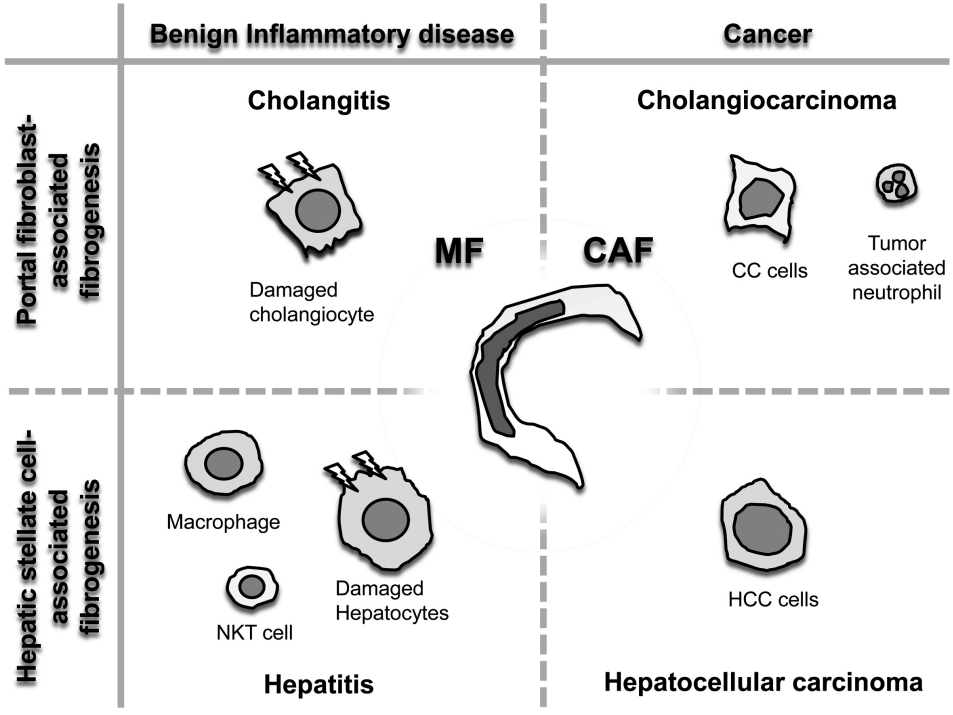

Fig. 1. Key players activating stromal fibroblasts in benign liver disease or malignant liver disease. Cholangitis and hepatitis have different mechanisms to make MFs. Previous studies showed that how CAFs are activated in CC is different from how they are in HCC. MF: myofibroblast; CAF: cancer-associated ficroblas; CC cells: cholangiocarcinoma cells; NKT cell: Natural Killer T cell.

of biliary tract, immune cells have been more emphasized than other stromal components. How MFs are activated and support the progression of biliary fibrosis in this situation needs further investigation upon the individual function of HSCs and PFs.

What makes CAFs in cholangiocarcinoma?

CAFs constitute a substantial cell population of the tumor stroma in many carcinomas, and the proportion of them is variable (Kalluri and Zeisberg, 2006). While activated fibroblasts are essential for normal wound healing, CAFs have been shown to promote the development of tumor including ICC (Bhowmick et al., 2004; Okabe et al., 2009, 2011a,b, 2012; Hanahan and Weinberg, 2011). Previous evidence indicates that CAFs are recruited by cancer cell-secreted factors, such as TGF-β and PDGF (Bierie and Moses, 2006; Kalluri and Zeisberg, 2006). Both of them are well-known pivotal inducers in hepatic fibrogenesis as described so far in this review.

Human CC cell lines produce PDGF-D which induces the recruitment of fibroblasts via the activation of ERK1/2 and JNK *in vitro*. CC cells in human express PDGF-A and -D, whereas CAFs strongly express PDGFRβ (Cadamuro et al., 2013). On the other hand, CAFs in CC produced PDGF-BB and CC cells expressed PDGFRβ *in vitro* and in human. PDGF-BB stimulates Hedgehog (Hh) signaling and promotes CC cell survival dependent on a cyclic adenosine monophosphate (cAMP)-dependent kinase (PKA) *in vitro*. Of note, Hh-signaling inhibition decreased growth and metastasis *in vivo* (Fingas et al., 2011; El Khatib et al., 2013). Seemingly this inhibition of Hh signaling works in both CC cells and CAFs. Taken together, these observations indicate that both PDGF and Hh signaling is crucial for CC-CAF interaction. Claperon et al. recently reported that CAFs activate cancer cells by secreting epithelial growth factor (EGF) and that activation of EGFR signaling also triggers TGF-β1 production in cancer cells (Claperon et al., 2013). Tumo-stromal interaction is mediated by EGF and TGF-β1.

We explored the crucial factors stimulating tumor-CAF interaction and identified that IL-1β derived from HSCs induced CXCL5 secretion in CC cells, which promoted migration of CC cells in the autocrine fasion (Okabe et al., 2012). This chemokine does not activate HSCs, but recruites CD66b expressing cell which is known as TAN in ICC. TANs might play an important role in the interaction between CC and CAFs as a linker (Li et al., 2011). TAMs are also activated in the tumor microenvironment, and might support tumor progression (Hasita et al., 2010). However, the evidence about the role of other stromal components in CC development is still limited. A rat CC model which mimics human CC seems to be the ideal tool to confirm them (Sirica et al., 2008).

HSC-associated liver fibrogenesis

Hepatocyte injury caused by viral hepatitis, alcoholic liver disease, or non-alcoholic steatohepatitis (NASH) induces oxidant-stress-mediated necrosis. Subsequently necrotic hepatocytes and Kupffer cells that engulfed those cells release free radicals, cytokines, and intracellular constituents, leading to HSCs activation. In alcoholic liver disease, Kupffer cells have been implicated as mediators through their release of TNF-α, TGF-β, free radicals, and other inflammatory mediators (Wheeler et al., 2001). TNF-α induces apoptosis of hepatocytes, which causes the activation of HSCs (Canbay et al., 2004). Experimental findings demonstrated that co-culture with Kupffer cells induced the activation of HSCs via the production of IL-6 and

Table 2. Crucial factors regulating MFs in chronic liver disease and CAFs in hepatocellular carcinoma.

Factors	Source of cellular type	Disease/material	Species	Ref
Hepatic stellate cell-associated liver fibrogenesis				
Oxidative stress	Hepatocytes	ALD	Hu	Day CP, Gastroenterology 1998
TNF-α, TGF-β	Kupffer cells	ALD	Rat	Wheeler MD, 2001
Leptin, TGF-β	Endothelial cells, Kupffer cells	Thioacetamide	Rat	Ikejima K, Gastroenterology 2002
HCV infection	-	HCV-CH/LC	Hu	Schuppan D, Cell Death and Diff. 2003
Apoptotic hepatocytes	-	Cirrhosis	Hu, Ms	Canbay A, Hepatology 2004
IL-6, H_2O_2	Kupffer cells	Co-culture	Rat	Nieto N, Hepatology 2006
LPS (TLR4)	-	ALD	Ms	Hritz I, Hepatology 2008
RPS5	Hepatic stellate cell	Cirrhosis	Ms, Hu	Xu WH, Hepatology 2014
Hepatocellular carcinoma				
CTGF, TGF-β	Hepatocellular carcinoma	Xonograft	Hu	Mazzocca A, Hepatology 2010
LPA	Hepatocellular carcinoma	Tumor tissue	Hu	Mazzocca A, Hepatology 2011
PDGF-C	Hepatocytes	Tg-mouse	Ms	Wright JH, Int J Cancer 2014
Liver metastasis				
VASP	Colorectal cancer	Metastasis	Ms	Tu K, Hepatology 2015

ALD: alcoholic liver disease; Tg: transgenic; LPA: lysophosphatidic acid; RPS5: ribosomal protein S5; VASP: Vasodilator-stimulated phosphoprotein.

H_2O_2 (Nieto, 2006). In the mouse model of alcohol-induced liver injury, Toll-like receptor 4 (TLR4) deficient mice showed protective phenotype (Hritz et al., 2008). TLR4 activation in HSCs using lipopoly-saccharide (LPS) *in vivo* recruits Kupffer cells and thereby promotes TGF-β-induced profibrogenic signals (Seki et al., 2007). In HCV-induced liver injury, it remains debatable how the liver fibrosis develops. HCV proteins seem to modulate apoptosis via steatosis, which causes activation of HSCs. HSCs might be directly activated by HCV infection, which leads to production of reactive oxygen species and TGF-β (Schuppan et al., 2003). Steatohepatitis is pathologically characterized by ultrastructural mitochondrial disorders such as linear crystalline inclusions in megamito-chondria, which is absent in most patients with simple steatosis and in healthy subjects (Sanyal et al., 2001). Insulin resistance leads to the accumulation of fat within hepatocytes, and subsequently increased intrahepatic levels of fatty acids provide a source of oxidative stress (Day and James, 1998). Mitochondrial injury-related reactive oxygen species might cause lipid peroxidation, cytokine induction, and the induction of Fas ligand, leading to activation of HSCs. In addition, leptin, which is a circulating adipogenic hormone, might amplify the fibrogenic activity, because HSCs enhanced fibrogenic signaling through the leptin receptor (Ikejima et al., 2002).

Recent accumulative evidences showed Hedgehog (Hh) signaling, master regulator of organ development, is important for fibrogenic liver repair (Choi et al., 2011; Omenetti et al., 2011). Several types of liver cells are capable of producing Hh ligands, including hepatocytes, cholangiocytes, HSCs, NKTs, and sinusoidal endotheial cells. Proapoptotic stimuli triggers mature hepatocytes to produce Sonic hedgehog (Shh) but they are not capable of responding to it (Sicklick et al., 2005). HSCs are responsive to Hh and they express it in the autocrine fasion, and inhibition of the Hh pathway can reduce liver fibrogenesis in mouse (Yang et al., 2008; Chen et al., 2012b).

The intracellular molecule, ribosomal protein S5 (RPS5), was introduced to have a potent inhibitory effect on HSC activation via reduction of Akt phosphorylation and subsequent dephosphorylation of GSK3β or P70S6K (Xu et al., 2014).

What makes CAFs in hepatocellular carcinoma?

In HCC, the stromal component is not so abundant and stromal desmoplasia is not a typical finding. Nevertheless, recent evidences demonstrated that CAFs promote HCC. Lysophosphatidic acid (LPA), which is a potent bioactive lipid, is produced by hydrolysis of lysophosphatidylcholine by autotaxin (ATX). ATX is a mediator of tumor progression either as a motile factor or by producing LPA which is reported to induce proliferation, apoptosis, migration, and invasion in HCC (Park et al., 2011). LPA is secreted by HCC cells and promotes transdifferentiation of myofibroblasts by the paracrine mechanism. Intriguingly, LPA was clearly shown to be a therapeutic target for tumor-CAFs interaction in HCC (Mazzocca et al., 2011). A recent study using transgenic mouse suggested that PDGF-C overexpressing hepatocyte causes activation of HSC which in turn produces HGF and cytokines, resulting in the development of HCC (Wright et al., 2014). Another study showed that TGF-β dependent down-regulation of CTGF diminished tumor growth and metastasis by inhibiting CAFs proliferation (Mazzocca et al., 2010). We previously showed that TGF-β signaling is critical for HCC development, and therefore a therapeutic strategy targeting TGF-β signaling is considered to be reasonable (Mima et al., 2012). However, patients with CC depending on TGF-β signaling show better prognosis than others (unpublished data). The mechanism by which CAFs get activated in HCC should be addressed carefully.

CAFs in metastatic liver tumor

Vasodilator-stimulated phosphoprotein (VASP) regulates TGF-β mediated HSC activation procell and tumor growth in a metastasis model with colorectal cancer. VASP forms protein complexes with TGF-β receptor II (TβRII) and Rab11, a Ras-like small GTPase and key regulator of recycling edosomes (Tu et al., 2015).

Conclusions and future perspectives

Genome wide analysis using a deep sequencing has been reported in many malignancies in the past 5 years, contributing to a deep comprehension of genomic landscape (Cancer Genome Atlas, 2012a,b; Lee et al., 2012). Genetic variations of tumor have been unveiled to be more intricate than we expected. Nonetheless, it is largely unknown how genetic background of tumor accounts for the mechanism by which CAFs get activated. We are now addressing the relevance of genetic variation of liver cancer cells to characteristics of stromal cells. Although CAFs might have a pivotal role among stromal cells in liver cancer development, it is certain that they are a supporting player in tumor progression. Strategy to target tumor-stromal interaction should be contrived based on how the leading player, cancer cell, orchestrate it. In fact, depletion of CAF seems to be the best strategy for cholangiocarcinoma, and a recent study suggested that navitoclax, which is a cytotoxic agent, inhibited tumor formation of cholangiocarcinoma via apoptosis of CAFs using a rat model (Mertens et al., 2013). On the other hand, depletion of CAFs in tumor stroma unexpectedly accelerates the malignant potential of pancreatic cancer, as Kalluri et al has showed using a mouse model (Ozdemir et al., 2014). We have to better understand the mechanism of tumor-CAF interaction, the way cancer cells controll the behavior of CAFs, and therapeutic

molecules regulating the interaction.

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Accepted September 23, 2015