A Review on Liver Fibrosis: It’s Pathogenesis, Resolution and Experimental Models

Keywords: Liver Fibrosis, Hepatic Stellate Cell, Cytokines, Animal Models

ABSTRACT

Liver fibrosis is caused by various etiologies including metabolic diseases, alcohol abuse and viral infection and end stage of chronic liver disease. It is defined as the excessive accumulation of extracellular matrix that is mainly including collagen. Activated hepatic stellate cells have been identified as the main collagen-producing cell in the liver and are the key mediator of fibrogenesis. Advanced liver fibrosis can progress into more severe stages known as cirrhosis and further to hepatocellular carcinoma. This review gives an update on the current in vivo and in vitro animal models used in the experimental liver fibrosis. This article reviews herbal medicine that has demonstrated activity against liver fibrosis through inhibition of cytokine production. In this review, we examine the involvement of diosgenin in the treatment of liver fibrosis by reducing the production of collagen, α-SMA and reduced expression of TGF-β1 by inhibiting hepatic stellate cell proliferation.
INTRODUCTION

Liver fibrosis is an imbalance of synthesis and decomposition of extracellular matrix (ECM) including predominantly type I collagen, and decreased in the remodeling of the extracellular matrix proteins which disrupts the normal structure of the liver [1, 2, 3]. Liver fibrosis cause due to alcoholic liver diseases (ALD), nonalcoholic fatty liver diseases (NAFLD), chronic hepatitis B (HBV) and hepatitis C (HCV) [4-6]. Increased deposition of ECM and formation of an abnormally large amount of scar tissue in the liver called as liver fibrosis [7]. In fibrosis causes scarring of liver cells, if more of the liver becomes scarred, called as liver cirrhosis. Type I and type III are the types of collagen that make up scar tissue, also called as fibrous tissue. Classically, hepatic stellate cells (HSCs) play a vital role in the pathogenesis of liver fibrosis and consisting of 15% of the liver cell mass [8, 9]. The activation of hepatic stellate cells is most important during the progression of liver fibrosis. Hepatic stellate cells are vitamin A storing cells and proliferate, lose their vitamin A and causes the transformation to myofibroblast (activated HSCs) during the progression of liver fibrosis that produces extracellular matrix proteins [10, 11, 12]. Some cytokine plays important role in the hepatic stellate cells activation and regulation of ECM proteins formation [13, 14]. Macrophages such as kupffer cells are the major source of these cytokines. Matrix metalloproteinase (MMP) degrade the ECM proteins, but in fibrotic liver tissue metalloproteinase’s (TMMPs) inhibits the MMP and play a major role in fibrogenesis. Therefore, the inactivation of activated HSCs is important for the recovery from hepatic fibrosis. Sometimes fibrosis can reverse if their cause is identified quickly. This review will integrate current knowledge about the nature of fibrosis with recent advances in elucidating its pathophysiology [15, 16].

PATHOPHYSIOLOGY OF LIVER FIBROSIS

Hepatic fibrosis results in wound healing response of the liver due to repeated injury [17]. Liver fibrosis or liver injury involves an acute and chronic response [18]. When the acute liver injury is not severe, then neighboring hepatocytes are able to replace necrotic cells. If the hepatic injury persists, hepatocytes become substituted by the extracellular matrix [19, 20]. ECM removing matrix metalloproteinases activity decreased due to an over-expression of their specific inhibitor Tissue inhibitor of metalloproteinases (TIMPs). The degradation of excessive extracellular matrix enhanced, by increasing the activity of MMPs and decreasing with their inhibitors, TIMPs [3, 21].
Hepatic stellate cell activation:

The activation of HSCs consists of two major phases: initiation and perpetuation. Initiation occurs in the cellular changes of gene expression and in HSCs. Perpetuation occurs when stimuli continue the HSCs activated phenotype and generate fibrogenesis.

a) Initiation of hepatic stellate cell activation

Activation of the hepatic stellate cell (which store fats) in the liver, initiates liver fibrosis. Activated HSCs are contractile, proliferating and cause loss of vitamin A storage. HSCs are vitamin A storing cell and are the main cells involved in the production of extracellular matrix seen in liver fibrosis.

Fig 1: HSC activation pathway

Production of an excessive amount of abnormal matrix (consisting of collagen) involved in the aggregation of kupffer cells, injured hepatocytes, and platelets. Resulting in inflammatory mediators (TGF, PDGF, ROS, IGF and ET-1) are released. Endothelin-1 is the stimulator of myofibroblasts; it helps to increased portal veins resistance and it increases the concentration of abnormal matrix in the liver. HSCs undergo transdifferentiation of vitamin A-storing cell to myofibroblast-like cell and increased proliferation, production of inflammatory cells (kupffer cells). TGF-β1 is the most important cytokine stimulating
fibrogenesis in the stellate cells. These cells produce excessive amounts of an abnormal matrix consisting of collagen. In HSCs, TGF-β stimulates the synthesis of ECM proteins and inhibits their degradation. Activation stimuli triggering HSC activation and these are originating from the injured hepatocytes. Hepatocytes are the major source of lipid peroxides in injured liver, thus it increases ROS production and stimulating the expression of collagen I. Proliferation of HSCs and collagen synthesis can also induce by ROS generation by cytochrome P450 2E1 (CYP2E1) in hepatocytes, which is normally seen in alcoholic liver disease (ALD). Hepatocellular apoptosis after injury may also contribute to liver fibrosis. Engulfment of the apoptotic bodies by HSCs induces the intracellular signaling cascades that promote the expression of collagen type I secretion and TGF-β 24. kupffer cells are one of the main sources of TGF-β1 production which leads to the transformation of stellate cells into myofibroblasts. This TGF-β act with platelet-derived growth factor (PDGF) to promote collagen I expression. Kupffer cells activation also facilitates HSC activation by secretion of ROS and TGF-β, this cytokine causes collagen deposition in the liver. The pathogenesis of liver fibrosis associated with the alcoholic liver disease, non-alcoholic fatty liver disease and hepatic stellate cells mechanism involved in liver fibrosis [3, 26].

Fig.2. Normal liver and fibrotic liver

In chronic liver diseases, hepatocytes undergo apoptosis; activate kupffer cells or inflammatory cells and releases fibrogenic factor in the liver. HSCs proliferate and secrete huge amounts of extracellular matrix proteins. Contraction of HSCs causes increased
resistance to the blood flow in the hepatic sinusoid and also increases expression of the cytoskeletal protein α-smooth muscle actin (α-SMA). Apoptosis of the injured hepatocytes stimulates the fibrogenic action of myofibroblast cells. In the liver, epithelial cells stimulate the accumulated myofibroblasts to initiate the collagen deposition around the damaged bile duct. Inflammatory cells (infiltrating lymphocytes) promote activation of HSCs to secrete collagen can resulting in changes in the composition of the ECM can stimulate fibrogenesis (Figure 2) [21].

b) Perpetuation of hepatic stellate cell activation

In this second step, HSCs become more proliferative and contractile, leading to enhanced production of ECM proteins and acquire a more myofibroblastic phenotype. The proliferative stage that accompanies activation of HSCs is governed by PDGF, which signaling underlies the activation of the mitogen-activated protein kinase involved in HSC growth and chemotaxis [27]. In the proliferative stage, the acquirement of contractility is a determinant in intrahepatic vascular resistance during liver fibrosis [28]. Furthermore, the contractibility can also be regulated by nitric oxide synthase, which is involved in the relaxation of HSCs and that can be inhibited by TGF-β [29]. The maintenance of these ECM proteins in the fibrotic liver is due to the interaction between MMPs and TIMPs secreted by activated HSCs, resulting in the deterioration of the healthy ECM in liver [30].

In chronic liver disease, activated HSCs play a role in the inflammatory response which can enhance hepatocellular apoptosis, and perpetuate the stimuli of fibrogenesis [31]. In this environment, activated HSCs are characterized by the expression of chemokines promote the migration of activated HSCs to the site of injury, thereby boosting the inflammatory response [32]. Other chemokines, such as monocyte chemo-attractant protein-1 and chemokine C-XC receptor 3, are also involved in cell chemotaxis [33]. In difference; activated HSCs secrete pro-inflammatory cytokines that behave as chemoattractants in the recruitment of inflammatory cells. This production of pro-inflammatory cytokines is promoted by the presence of lipopolysaccharide secreted by gut bacteria upon binding to Toll-like receptor 4 and by the ethanol consumption. The chronicity of the injury allows full transdifferentiation of HSCs into myofibroblastic cells, which interact with a number of cells to enhance scar formation, the reduction of liver blood flow [34, 35].
RESOLUTION OF LIVER FIBROSIS

Resolution of liver fibrosis has been demonstrated in human and animal models that liver fibrosis can be considered a bidirectional process and can be reversible. The mechanisms regulating HSC survival and apoptosis in the resolution of liver fibrosis are summarized in figure 3. In liver other inflammatory cells and liver myofibroblasts involved in the fibrotic process, including kupffer cells and secrete a matrix-degrading MMPs [36]. These enzymes degrade collagen and other matrix molecules. Molecular studies of the mRNA expression for these enzymes (including those with collagenolytic activity) have demonstrated that they are expressed in the liver and cirrhosis, but their activity is limited by inhibitors (TIMP-1 and TIMP-2). In the recovery process, TIMP-1 and TIMP-2 expression decrease rapidly while matrix degrading MMPs continue to be expressed, resulting in increased consequent matrix degradation and collagenase activity within the liver.

Fig 3: Resolution of liver fibrosis

The kupffer cells and inflammatory cells activate thereby increases the level of collagen and TIMPs by decreasing collagenase enzyme activity. Collagenases are the enzyme that breaks the peptide bond in collagen. During liver injury when HSCs are activated in the normal wound-healing response, HSC apoptosis is forestalled. When the harmful stimulus has withdrawn the loss of these survival factors causes the activated HSCs undergoes apoptosis, which facilitates the remodeling process by removing a cellular source of TIMP and
Resolution process was characterized by apoptosis of HSCs, mainly at the margins of fibrotic septa and residual septa, which had not remodeled after one year. Therefore incomplete resolution may be a result of qualitative changes in the hepatic matrix and cause failure of HSC apoptosis. In resolution process decrease the levels of collagen and increases collagenase enzyme in the liver. Death receptor activation and abnormal matrix degradation favor hepatic stellate cell activation. A number of studies have investigated some agents which stimulate HSC apoptosis. For example, the addition of gliotoxin to activated human and rat HSCs results in morphological alterations characteristics of apoptosis and caspase 3 activations [4, 37].

CYTOKINES INVOLVED IN LIVER FIBROSIS

Different types of cytokines regulating the inflammatory response to modulate hepatic fibrosis. TGF-β1 play the major role in liver fibrogenesis, and cause HSCs transition to myofibroblast-like cells, stimulates the synthesis of ECM including collagen by inhibiting their degradation. Liver fibrogenesis regulates when cytokines with vasoactive properties [13]. Nitric oxide is the vasodilator exert antifibrotic effect while vasoconstrictors such as angiotensin II, norepinephrine has the fibrotic effect and Endothelin-1 are the powerful vasoconstrictor, it stimulates liver fibrogenesis [38]. Vasoconstrictor, angiotensin II induces inflammation in hepatic cells and stimulates the fibrogenic effect in activated HSCs, by inducing cell proliferation and collagen synthesis [39].

Adipokines, which are cytokines, regulate hepatic fibrogenesis mainly derived from the adipose tissue including leptin, adiponectin, resistin [40]. Following liver damage, produce pro-inflammatory cytokines by, kupffer cells, HSCs, hepatocytes, lymphocytes, natural killer cells in the liver [41]. Cytokines can activate the Janus kinase-signal transducer pathway in the liver, including interferon-α, IFN-β, IFN-γ, interleukin-6 (IL-6) and IL-22. When extracellular cytokine protein binds to its corresponding transmembrane receptor complex, the JAK-STAT pathway is activated including JAK 1, 2, 3 [42].

GENE EXPRESSION

Changes in the pattern of gene expression during HSC activation:

HSC activation is associated with several changes in the pattern of cellular gene expression. Particularly, in relation to the fibrogenic nature of the activated HSC, is the induction of type
I collagen gene expression. By using several molecular techniques, including suppression subtractive hybridization (SSH) methodology, microarray analysis and differential display PCR (DD-PCR), it has become clear that the pattern of gene expression markedly changes during HSC activation. Below, some of the most important changes in the pattern of gene expression that occurs during HSC activation are discussed (Table 1).

In comparison to inactivated hepatic stellate cells activated HSCs exhibit a marked difference in their gene expression profile.

**Table 1: Expression changes during activation of the hepatic stellate cell**

<table>
<thead>
<tr>
<th>Increased expression during HSCs activation</th>
<th>Decreased expression during HSCs activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle α-actin (α-SMA)</td>
<td>Liver carboxylesterase</td>
</tr>
<tr>
<td>Transforming growth factor-β (TGF-β)</td>
<td>AMP deaminase</td>
</tr>
<tr>
<td>Stellate cell activation-associated protein (STAP)</td>
<td>Protein inhibitor of activated STAT-1 (PIAS-1)</td>
</tr>
<tr>
<td>α1 (I), α2(I), α1(III) procollagen</td>
<td>P19 (cyclin-dependent kinase inhibitor)</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinases-1 (TIMP-1)</td>
<td>A-1 serine protease inhibitors 3 and 4</td>
</tr>
<tr>
<td>Intercellular adhesion molecule-1 (ICAM-1)</td>
<td>Betaine homocysteine methyltransferase</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Type II DNA topoisomerase-β</td>
</tr>
</tbody>
</table>

**Type I collagen**

The collagen family represents a group of ECM proteins involved in providing structural support to connective tissue and forming the basement membrane of different types of organs, including the liver. There are 27 different collagen proteins have been identified, however, from these collagen type I, III and IV are most associated with liver fibrosis. Increase in expression and deposition of type I collagen are the most important as an aspect of liver fibrosis and expression of type III collagen is also increased. The increase in type I collagen is reflected by the increase in levels of α1 (I) and α (I) collagen mRNA. Type I collagen is consisting of two α1 chains and α2 (I) chain and the transcription of these two genes expression is coordinately regulated. Expression of type I collagen is very low in inactivated hepatic stellate cells; however, following a fibrogenic stimulus, the HSCs...
undergo a complex, increased production and secretion of type I collagen, also increased synthesis of both α1 (I) and α2 (I) collagen mRNA expression. The induction of transcription factors plays an important role in the transcriptional activation of collagen type I, which may occur through several transcription factors. PPAR-γ is one such important factor whose activity is decreased in activated HSC and its expression inhibits HSC activation\(^{[45]}\).

Activator protein-1 (AP-1) which includes the Jun family of proteins (c-Jun, JunB, JunD) and Fos family members (c-Fos, FosB, Fra1, Fra2) regulates the MMP gene expression in liver\(^{[46]}\). Kruppel-like transcription factors (KLFs) such as Sp1, and BTEB 1 increase expression of α1 (I) collagen and TGF-β in activated HSCs. TGF-β enhances α1 (I) and α2 (I) collagen gene expression through an interaction between Sp1 and other components of the TGF-β response element complex\(^{[47]}\). Changes in α2 (I) collagen gene expression occur by post-transcriptional modification of protein interacting with Sp1. Type I collagen biosynthesis is a complex process and that is regulated at both the protein levels and mRNA and many of these regulatory processes are different in the inactivated versus activated HSC phenotype\(^{[48]}\).

**Other ECM proteins**

In HSC activation not only type I collagen gene is over-expressed also increases their synthesis of collagen type α1 (III), α1 (IV), associated with fibrotic liver\(^{[49]}\). In addition, a major source of ECM production activated HSCs also show increases in expression of MMP-2 and MMP-9, which serve as modulators of ECM accumulation and inhibitor of MMP, the tissue inhibitors of matrix metalloproteinases (TIMPs). The interaction between extracellular matrix synthesis and degradation plays a central role in extracellular homeostasis and cause alterations in ECM integrity that can result in the establishment of liver fibrosis. The glycoprotein fibronectin, which is involved in cell adhesion, migration, and formation of interstitial connective tissue, is induced HSC activation and the protein has also been shown to induce HSC activation\(^{[50, 51]}\). Protein kinase C-mediated signaling pathway partially regulated fibronectin synthesis\(^{[52]}\). In fibrotic liver, various types of other glycoproteins, including dystroglycan, vitronectin and secreted protein acidic and rich in cysteine (SPARC) levels are elevated\(^{[53, 54, 55]}\).
STIMULI OF LIVER FIBROSIS

Alcoholic liver disease (ALD)

Excessive alcohol consumption is one of the most common causes of liver fibrosis. Alcohol increases lipid peroxidation by enhancing the production of reactive oxygen species (ROS) and decreasing the level of endogenous antioxidants. The breakdown of alcohol within hepatocytes produces acetaldehyde and ROS, both of which have pro-fibrotic properties on HSC via paracrine mechanism. This acetaldehyde stimulates collagen expression in fibroblast. Activation of c-jun nuclear-kinase (JNK) involved in mediating the increase in collagen expression by acetaldehyde by increasing transcription factor binding to the collagen promoter. Elevated level of collagen gene expression reduced by inhibiting JNK activity[56].

Oxidative stress development is strongly associated with ethanol-induced liver fibrosis. The fibrogenic effect of ethanol is generated ROS intermediates by cytochrome P450 2E1 (CYP2E1) [57]. ROIs increases extracellular matrix production and directly influence HSCs. The formation of ROI and generation of lipid peroxidation reduces by inhibiting CYP2E1 activity [58, 59]. ROIs generated from ethanol metabolism as well as NADPH oxidase present in kupffer cells also produce ROIs. ROIs can activate kupffer cells by increasing the production of both pro-inflammatory and profibrogenic cytokines [60].

Fatty liver disease

NAFLD

Nonalcoholic fatty liver disease is defined as the accumulation of more than 10% fat in the liver without any primary cause such as ALD [61]. NAFLD is the most common chronic liver disease, affecting up to 30% population n the European countries and the United States [62]. The most common form of NAFLD is a nonserious condition known as fatty liver. NAFLD is also associated with reported patient histories including hyperglycemia, hyperuricemia, hypertension and polycystic ovarian syndrome. Prevalence of NAFLD has been associated with metabolic syndrome and insulin resistance is an intrinsic defect in NAFLD [63].
NASH

Nonalcoholic steatohepatitis is characterized by a spectrum of apoptosis, necrosis, and fibrosis. Histologically, steatohepatitis is characterized by steatosis with lobular inflammation and hepatocytes ballooning in the presence or absence of fibrosis. In NASH, fat accumulation is associated with inflammation of liver cells and different stages of scar formation. NASH is also characterized by neutrophil inflammation and elevated serum alanine transaminase (ALT) and aspartate transaminase (AST) levels \(^{64}\). In some patients, shows symptoms such as abdominal pain, fatigue, right upper quadrant pain \(^{65}\). Progression to steatohepatitis and fibrosis depends on inflammatory pathways activated by free fatty acids (FFA), cytokines, mitochondrial dysfunction and oxidative stress. FFA levels increases during fat accumulation, activate PPAR-\(\alpha\), which result in FFA oxidation and the formation of reactive oxygen species (ROS) with oxidative stress causing hepatocytes damage. FFA induces the production of pro-inflammatory cytokines (tumor necrosis factor-\(\alpha\), inducible nitric oxide synthase, interleukin-6) acts through NF-kB \(^{66}\). Antioxidants such as N-acetylcysteine, betaine has shown potential results on hepatic serum enzyme levels and did administration of vitamin E to children with NASH \(^{67}\). The PPAR-\(\gamma\) ligand pioglitazone, which acts by reducing hepatic steatosis and prevented liver fibrosis in the rat model of NASH, and also prevented HSC activation and decrease type I procollagen mRNA levels in vitro \(^{68}\).

IN VIVO MODELS OF LIVER FIBROSIS

Carbon tetrachloride

Carbon tetrachloride has been widely used to experimentally induce hepatotoxicity in the rodent. A single dose of CCl\(_4\) leads to steatosis and necrosis, although prolonged administration of CCl\(_4\) leads to liver fibrosis, cirrhosis \(^{69}\). CCl\(_4\) is metabolized in the liver by cytochrome P450 (CYP450) to the trichloromethyl radical, which is further involved in the lipid peroxidation process \(^{70}\). That contributes to the activation of kupffer cells and induction of an inflammatory response and it is associated with the production of several cytokines, which promote activation of hepatic stellate cells, hence produce liver fibrosis. CCl\(_4\) model can be applied to both rat and mice \(^{24}\). However, mice are most preferred; because of mice have a higher metabolic rate of CCl\(_4\) as compared to a rat. In the experiment,
CCL₄ is injected intraperitoneally twice a week for 28 days [14]. Otherwise, CCL₄ can be administered orally, subcutaneously and through inhalation 2 times per weeks [70].

**Thioacetamide**

Thioacetamide (TAA) is a second widely used model for the induction of experimental liver fibrosis, liver tumor, and acute liver failure. Though TAA itself not hepatotoxic, its reactive metabolites covalently bind to lipids and proteins, thus causing centrilobular necrosis and oxidative stress [71]. This bioactivation process, catalyzed by CYP450 isoenzyme, result in the formation of Thioacetamide sulfur dioxide, it may involve in down-regulation of enzymes involved in fatty acid β-oxidation. Moreover, resulting in severe oxidative damage associated with HSC activation. Thioacetamide is administered intraperitoneally in doses between 100 and 200 mg/kg three times per week for 6 weeks. These animals show an enlarged liver with mild inflammatory cell infiltration along with elevated alanine aminotransferase and aspartate aminotransferase serum levels. When Thioacetamide administered orally, in higher doses of 200 to 300 mg/kg body weight is used for 16 weeks. When orally administered 300 mg/l in drinking water to C57BL/6 mice require 2 to 4 months to develop significant fibrosis [24, 72].

**Ethanol**

Alcohol induces fatty liver and can progress to steatohepatitis, fibrosis, and cirrhosis lead to hepatic failure. Ethanol is primarily metabolized by alcohol dehydrogenase and CYP450 enzymes. This process is involved in the production of ROS, lipid peroxidation and increased collagen synthesis. Together, these mechanisms induce inflammation and the activation of HSCs. Female mice were more susceptible to ethanol-induced alcoholic liver disease (ALD) than a rat. ALD causes imbalanced in the immune response that results in the increased production of pro-inflammatory cytokines. In ALD Tumor necrosis factor-α (TNF-α) is the major pro-inflammatory cytokine, involved in inflammatory response [73]. The Tsukamoto-French intragastric feeding model produces hepatic injury and inflammation with the onset of mild fibrosis, but TF intragastric feeding model failed to develop liver fibrotic stages. Due to these limitations, new techniques such as the combination of ethanol administration with the second stimulus, including specific diets, CYP450 inducers, hormones, pharmacological agents have been introduced [74].
Dimethylnitrosamine and diethylnitrosamine

These are the carcinogenic compounds that are commonly used to experimentally induce liver fibrosis in animals. DMN and DNM act as a liver poison producing ROS, triggering the development of centrilobular necrosis. In mice, B6C3F1 and C3H mice mostly develop tumors compared to C57BL mice and in rats, the R16 strain is most susceptible to these carcinogenic chemicals. DEN administered intraperitoneally to mice 40 and 100 mg/kg once per week for 2 weeks, orally administered to mice at a dose of 100μl/kg for 12 weeks and in rats, DEN administered weekly oral gavages of 5 ml of 1.5 %/kg during 3 to 11 weeks. DMN administered intraperitoneally 10μg/g 3 times per week for 3 weeks [24, 75].

IN VITRO MODELS OF LIVER FIBROSIS

Cell lines

Mouse cell lines

The first described hepatic stellate cell line is the GRX (murine cell line) was obtained from the liver of C3H/HeN mice infected by transcutaneous penetration of cercarias from the Schistosoma mansoni BH strain. In the culture medium, GRX cells show a myofibroblastic phenotype. When it is transferred to cell culture media containing insulin or retinol, GRX cells adopt a fat-storing phenotype. GRX phenotypes are able to express collagen type I, III, yet production of the different collagen types in the lipocyte-like phenotype is low. This lipolytic-like phenotype has the ability to metabolize retinol similar to HSCs. Therefore, the murine cell line is helpful in the study of lipid-related changes as also occurring during the liver fibrosis. However, both myofibroblast and lipocyte phenotypes should be considered activated states of HSC. A640-IS cells are HSC line is temperature sensitive, originated from male ICR mice that have been then transfected with large T-antigen of simian virus 40 (TSV40). The morphology is dependent on the temperature, at 33°C cells show a myofibroblastic feature and HSC –like morphology at 39°C. A640-IS phenotype produces collagens type I, III, α-SMA and fibronectin, desmin. Desmin is highly expressed at 39°C, however, α-SMA expresses in low-density cultures at both temperature. The M1-4HSC line isolated from male p19ARF null mice. M1-HSC cells emerge in two different phenotypes depending on the presence of TNF-β1. In the absence of TNF-β1, the M1-4HSC resemble quiescent HSCs. M1-4HSC cells adopt a more myofibroblastic morphology and produce pro-collagen I, α-SMA in the presence of TNF-β1. The immortalized cell lines JS1, JS2, and JS3
obtained from the isolated HSCs from Toll-like receptor 4-deficient and 88 deficient C57/B16 mice. These cells were then transfected with the cytomegalovirus promoter TSV40. Most recently, Col-green fluorescence protein (GFP) a new mouse cell line, has been described. These cells are HSCs isolated from transgenic mice expressing GFP under the control of collagen I gene promoter and treated with CCl4 for 8 weeks. These Col-GFP cells are considered for the screening of the potential anti-fibrotic drugs [24, 76].

**Rat cell lines**

HSC-T6 cell line was isolated from male Sprague-Dawley rats were transfected at day 15 of primary culture with the SV40T that was expressed under control of the Rous sarcoma virus promoter. The cytoskeletal markers of activated HSC including α-SMA, desmin, and vimentin are expressed in HSC-T6 and when cultured in media containing a high concentration of retinol, the cells form cytosolic lipid droplets and accumulate retinyl ester in the cytoplasm in the presence of retinol. In addition, 6 nuclear retinoid receptors, including retinoic acid receptor α, β, γ and retinoid X receptor α, β, and γ can be detected in HSC-T6 cells. HSC-T6 have been successfully used for identifying novel targets for liver fibrosis therapy and examining the signaling pathways involved in collagen expression. This cell line was also evaluated to express adhesion molecules, proliferative, and inflammatory genes in the presence of lipopolysaccharide.

PAV-1 are immortalized cells with myofibroblastic appearance isolated by amplification of a colony that was obtained by spontaneous immortalization of isolate primary HSC isolated from an over 8-month-old male Wistar rat. These cells express the same HSC activation markers as HSC-T6 cells, but lack production of collagen. PAV-1 cells line also express RARα and RXRα and used in ALD research. Retinol metabolism in PAV-1 cells is disrupted, in the presence of ethanol, thereby decreasing levels of lipid droplets in the cytoplasm, in turn leading to a more active phenotype. Therefore, PAV-1 cell line is used for studying the role of free fatty acids in ALD.

The biliary stellate cell (BSC) line isolated from rats with biliary liver fibrosis. The BSC clones generated by spontaneous immortalization include BSC-C10, which expresses markers of HSC activation, such as procollagen and α-SMA. This line has been used to investigate the molecular pathways involved in HSC activation.
MFBY2 are immortalized cells have been isolated from a cirrhotic rat liver and show HSC activation markers, including α-SMA, fibronectin, collagen type I and III, neural cell adhesion molecule (N-CAM). When MFBY2 cells transfected with an adenovirus containing the terminal latency-associated peptide of TGF-β1, MFBY2 cells present an HSC-like cell shape. HSC-PQ cell line isolated from primary rat HSC that were separated from an adult male Sprague-Dawley rat. Immunohistochemical analysis further exposed that the cells express collagen type I and III as well as laminin, desmin, fibronectin but lack collagen type I and IV [24, 76].

**Human cell lines**

The LI90 cell line was the first human HSC immortalized cell line found in the right liver lobe of 55-year-old Japanese female during cholecystectomy. LI90 cell produces collagen type I, III, IV, and VI, α-SMA, laminin, vimentin, and fibronectin. LI90 has a high proliferation rate and it has the ability to overgrow because of the lack of contact inhibition. Addition of vitamin A to the cell culture, LI90 cells form lipid droplets in the cytoplasm. This cell line constitutes a promising model for the description of drug targets in hepatic stellate cell activation and after a number of passages, these cells undergo senescence. This can be counteracted by the introduction of the human telomerase reverse transcriptase (hTERT) gene; resulting in a novel HSC line (TWNT-4) was generated. TWNT-4 cells express some HSC activation markers, including collagen and α-SMA.

GREF-X cells are HSCs, human myofibroblasts isolated from explants of a normal human liver and transfected with a coding sequence of polyomavirus large T antigen. These myofibroblast-like cells secrete MMP-2 and express collagen type I, III, IV and VI, fibronectin, laminin, vimentin as well as α-SMA. In addition, GREF-X cells are negative for indicative markers (i.e. CD68, cytokeratin, Von Willebrand factor). GREF-X retains the capacity to take up and esterifies retinol present in the cell culture medium. Although this cell line shows several anomalies of chromosome number, the cells are density-inhibited, require serum, do not grow in soft agar and are not able to induce subcutaneous tumors in nude mice [76].

The hTERT-HSC line was established to tackle the senescence of HSCs in the culture medium. This HSC cell line isolated from surgical specimens of normal human liver and infected with a retrovirus expressing human telomerase reverse transcriptase (hTERT) driven
by the cytomegalovirus (CMV) promoter. These cells produce platelet-derived growth factor receptor α (PDGFRα), IL6, IL8, IL10, and glial fibrillar acidic protein. hTERT cells maintain retinol uptake and metabolism capacity [24].

**Co-culture**

Co-culture system has been developed to study the interactions between different cells. Cell-cell interactions in the co-cultures are influenced by the extracellular environment. There are several methods have been developed to obtain the co-cultures consisting of two or more liver cell types. The cultures consisting of only one cell type are only limited use for studying liver fibrosis and HSCs activation. This monoculture of the cell does not consider the interaction between different cell types, hence which are critical for disease progression. Therefore, co-culture containing two cell types, have been developed. The co-culture consisting of primary HSCs is rare and primary hepatocytes. The hepatic stellate cell lines are used to developed co-culture with hepatocytes. These HSC-hepatocyte co-culture systems have been improved by including seeding between the 2 layers of ECM compound or by culturing in spheroids. In the spheroid co-culture of rat HSCs and hepatocytes, the expression of ECM proteins has been observed in abundant quantity, which supports to hepatocytes stability. Recently, the use of co-culture system based on HSC cell lines and hepatocytes demonstrated that cell to cell closeness is important to initiate a fibrotic response. Another one, co-cultures based on the primary HSC and kupffer cells reflect the role of immune cells in the regulation of fibrotic response, while co-culture system consisting of HSCs and endothelial cells have exposed the importance of HSCs in angiogenesis [77, 78].

**Precision-cut liver slices**

Recently, precision-cut liver slices model is potential for the study of HSCs activation and liver fibrosis. This model mostly used for the study of drug metabolism and their toxicity [79]. Various types of systems such as continuously submerged culture system and dynamic culture systems maintain many cellular interactions that occur in vivo. To obtain precision cut-liver slices, liver explants with a thickness of 100 to 250 μm and diameter of 5 mm, which allows nutrients and oxygen to diffuse. PCLS can be incubated in cell culture dishes, which may be incorporated in the dynamic organ culture system. In the continuously submerged system cut, liver slices are floating within the culture medium in 6, 12 and 24-wells plates or the liver slices placed on stainless-steel grid while the culture medium is
magnetically stirred. In the dynamic culture system, liver slices are alternately exposed to the gas phase and culture medium by pacing liver slices on inserts in 6-wells culture plate or glass vial, which is rolled, during incubation. irrespectively the liver slices incubated at $37^\circ$C in the presence of oxygen concentration varying between 20% oxygen (air)/5% CO$_2$, and 95% oxygen/ 5% CO$_2$ in a humidified incubator Incubation in 20% oxygen/ 5% CO$_2$ used for the short-term studies is sufficient to retain slice viability$^{[80]}$. In the experiment up to 24 h of incubation, PCLSs initiates fat accumulation in the liver at that point there is an activation of myofibroblasts. For the long-term incubation, the nutrient-rich culture medium is required. Although the liver slices are easily maintained in culture medium up to 48 h for a prolonged incubation of the liver slices carefully monitoring of the culture conditions is necessary. The liver slices viability may be also improved by inserting flow conditions in the culture system $^{[81]}$.

CONCLUSION

Liver fibrosis represents a wound healing response which is caused by various types of inflammatory stimuli and poses a significant health problem worldwide. Current evidence indicates that the development of liver fibrosis is a dynamic and bidirectional process. Fibrosis is characterized by an increase in the synthesis and deposition of ECM. The activated HSC has been identified as the cell type responsible for the increased production of ECM during fibrosis. For advanced end-stage liver fibrosis (cirrhosis) has the therapeutic option is liver transplantation, which poses a significant risk to the patient. Therefore the development of effective therapeutic approaches for the liver fibrosis is necessary.

REFERENCES


ls are key oxidants in alcohol

