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Liver Fibrosis: Mechanisms of Development, Experimental Models and Treatment Strategies.

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ABSTRACT

Fibrosis is a wound-healing process that occurs as a consequence of tissue injury. In the liver, persistent injury leads to deposition of extracellular matrix and can eventually result in a pathologic condition called liver cirrhosis, in which normal architecture of the liver is destroyed, and formation of regenerative nodules and septae takes place. Although, in humans, hepatic fibrosis can be caused by various factors, such as congenital, metabolic, parasitic, toxic, vascular or inflammatory, the molecular mechanisms that underlie fibrosis are basically the same. Activated hepatic stellate cells and portal fibroblasts are major contributors of extracellular matrix proteins during fibrosis. Inflammatory mediators play a significant role in the development of disease as well. There is currently a clear evidence that the most effective prevention of liver fibrosis is removal of causative agent, but the opportunities for development of effective treatment strategies are promising. The primary aim of this review article is to increase awareness of healthcare professionals, researchers and general public regarding liver fibrosis by providing information on etiology, pathogenesis, histopathology, and available and experimental treatment options for liver fibrosis; extensive description of current experimental models used for research of liver fibrosis is also present.

Keywords: liver fibrosis, extracellular matrix proteins, hepatic stellate cells, fibroblasts, liver fibrosis models, fibrosis prevention, fibrosis treatment.

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INTRDUCTION

Fibrosis is a wound healing process that occurs as a consequence of organ or tissue injury, and is an essential reaction of the body, especially in case of a large-sized lesion. But when a chronic injury takes place, this seemingly beneficial process will lead to scar formation with a potential decrease in function of affected organ or tissue.

In the liver, fibrosis is generally a slow process that takes a large amount of time to develop due to the high regenerative capacity of the liver. In other organs, such as kidney or lung, fibrosis is more rapid process and has more prominent effect on the organ function and patient quality of life and survival [1].

In the liver, persistent injury leads to deposition of extracellular matrix (ECM) and can eventually result in a pathologic condition called liver cirrhosis, in which normal architecture of the liver is destroyed, and formation of regenerative nodules and septae takes place [2]. In contrast to liver fibrosis, which is a reversible process, liver cirrhosis is irreversible, so the essential concept of prognosis improvement is to focus therapeutic efforts on modifying relatively reversible fibrosis phase [3].

EPIDEMIOLOGICAL AND CLINICAL DATA

In the United States, approximately 31000 people die from cirrhosis each year. It has been rated the ninth cause of death in the United States and is responsible for 1.2% of deaths. Many patients with the disease die in their fifth or sixth decade of life [4]. Some big trials investigated mortality rates of cirrhosis in 187 different countries from 1980 to 2010, it was discovered that from 1980 to 2010, number of deaths owing to cirrhosis increased from 676000 to over a million. Concurrently, the age standardized mortality rates dropped by 22% mostly due to drop in mortality in Western Europe, China and the United States. In places like Egypt, deaths in 45 to 54-year-old males were 20% of the time due to liver cirrhosis. While in France and Italy, mortality rate due to cirrhosis fell by 50% to 60%, in the United Kingdom, it increased by 30%. In Latin America, the highest rates belong to Mexico [5]. Cirrhosis has a 10-year mortality rate of about 34 to 66% although this largely depends on the cause of cirrhosis [6].

Some patients with liver cirrhosis appear not to have any symptoms related to their illness. These people are said to have a normal life expectancy. Others on the other had are said to have a myriad of symptoms which resemble those of an end-stage liver disease. These ones have a low life expectancy. These signs and symptoms result from a decrease in liver function such as inability to clot blood due to deficient clotting factor produced by the liver which causes a subsequent susceptibility to bruising. Others include signs of portal hypertension (an increase in the pressure within the portal vein) caused by variceal bleeding which could either be pre-, intra-, or post-hepatic. There symptoms include hepatomegaly which is enlargement of the liver, pain in the abdomen due to contact of the enlarged liver with the wall of the abdomen, ascites (fluid in the peritoneal cavity leading to swelling of the abdomen), jaundice or a yellow discoloration of the mucous membranes and skin due to excess deposition of the pigment bilirubin, skin telangiectasia in which the capillaries are dilated causing them to appear red or purple with a spider-like appearance, cyanosis, clubbing of the fingers, increased intracranial pressure leading to papilledema, hypertension and low heart rate, neurological symptoms which range from seizures to somnolence to confusion to coma. These neurologic deficits occur due to accumulation of the toxins that were supposed to be eliminated by the liver, getting to the bloodstream and hence the brain [7, 8].

MOLECULAR MECHANISMS OF LIVER FIBROSIS

Liver fibrosis can result from a diverse range of causes, among them viral infections, specifically with HBV and HCV viruses, alcohol abuse, nonalcoholic fatty liver disease and non-alcoholic steatohepatitis, hepatotoxic medications, biliary obstruction, autoimmune diseases, such as primary sclerosing cholangitis, primary biliary cholangitis and autoimmune hepatitis, metabolic disorders, such as hemochromatosis, Wilson's disease, alpha-1-antitrypsin deficiency, and other, less common etiologies.

The cellular components of the normal liver include hepatocytes, which are the major liver cells, resident macrophages, which are called Kupffer cells, and mesenchymal cells named stellate cells. There are two types of stellate cells - quiescent stellate cells, which store lipid droplets, and activated stellate cells, which

lose their lipid depositions after liver has been damaged, and are subsequently transformed into activated myofibroblasts, which are the major types of cells that deposit ECM proteins during fibrosis [2, 9].

The process of hepatic stellate cell activation can be broken down into several stages. The first stage, which is called initiation, is due to paracrine stimulation from damaged hepatocytes as well as Kupffer cells as a result of changes in ECM composition and injury to hepatocytes. Platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta) are the major cytokines in the liver that enhance stellate cell activation and proliferation [3, 9]. They are mainly produced by Kupffer cells, but can also be made by hepatic stellate cells. The overall effect of TGF-beta binding to its receptor is the induction of type 1 and 3 collagen transcription. It involves induction of the Ras signaling molecule pathway, leading to activation of the extra-cellular signal-regulated kinases (ERK)/mitogen activated protein kinase pathway [1, 10].

Injured hepatocytes and Kupffer cells are a great source of reactive oxygen species (ROS) including superoxide, hydroxyl radical, hydrogen peroxide, 4-hydroxy-2,3-nonenal and 4-hydroxy-2,3-alkenal. Apoptosis of hepatocytes, which is a feature of hepatic injury, leads to production of apoptotic bodies, which are being recognized by toll-like receptors (TLR9) on hepatic stellate cells, and subsequently leads to induction of fibrogenic activity by the stellate cells [1, 11, 12].

Sinusoidal endothelial cells participate as well in the process of initiation. Injured sinusoidal endothelial cells induce splice variant of cellular fibronectin (EIIIA), leading to activation of stellate cells, as well as to conversion of TGF-beta to its active form, which is fibrogenic [3, 13, 14].

The next step in stellate cell activation is perpetuation, characterized by phenotypic changes due to increased response to cytokines, as well as to changes in ECM composition. There is an increased expression of tyrosine kinase receptors and other cell membrane receptors to facilitate the response to cytokines with a change in subendothelial matrix to one abundant in fibril, with a formation of collagen. This ECM also activates the stellate cells through binding of collagen to discodin domain receptor 2 (DDR2) on stellate cells, speeding up their activation. This constantly affects behavior of hepatocytes, sinusoidal cells and especially stellate cells [3, 15, 16].

Phenotypic changes in activated stellate cells include proliferation, contractility, fibrogenesis, matrix degradation, retinoid loss, chemotaxis and attraction of WBCs. High-potency tyrosine kinase receptors, present on hepatic stellate cells, contribute to local proliferation of the stellate cells [3, 17].

Contractility of stellate cells is mainly conducted as a response to endothelin 1 (ET-1), which may be produced by the stellate cells themselves. They also facilitate proliferation. There are two G-protein-coupled receptors responsible for response to ET-1, ETA and ETB. These are expressed on both quiescent and activated stellate cells in contrast to receptor tyrosine kinases that are mostly induced. However, the effects of both receptors may differ based on the cellular activation [3, 18, 19, 20].

As mentioned earlier, TGF-beta is the major fibrogenic stimulus for stellate cells. TGF-beta knockout mice with acute liver injury showed significantly reduced collagen deposition [3, 21].

Activated stellate cells are an important source of matrix metalloproteinase-2 (MMP2) and stromelysin/MMP3 in liver injury, which are important matrix degraders. They degrade the normal subendothelial matrix facilitating its replacement by fibril-forming collagen-rich matrix, which on its own further activates stellate cells through the DDR2 receptor pathway, as mentioned earlier, leading to production of more MMP2 and MMP3 and creating a positive feedback loop system. In addition, activated stellate cells also inhibit tissue inhibitor of matrix metalloproteinases 1 and 2 (TIMP 1 and 2), leading to decreased degradation of collagen enhancing scar formation [3, 22, 23, 24].

PDGF and monocyte chemotactic protein (MCP-1) are important chemoattractants of activated stellate cells increasing their population in injured areas of the liver. Plasminogen activator is also required and this may be for the purpose of ECM degradation to enhance movement of the cells through the ECM.

The activated stellate cells also secrete chemotactic factors, including cytokines such as colony stimulating factor and MCP-1 which increase inflammatory cell migration into the liver.

It has been noted that CD8 lymphocytes are more fibrogenic towards stellate cells as compared to CD4 lymphocytes, explaining the increased fibrosis in HIV or HCV infections, where CD4/CD8 ratio is decreased [1, 25].

Natural killer cells, on the other hand, have the function of clearing activated stellate cells, which is enhanced by interferon gamma and retinoic acid and decreased by alcohol.

Although hepatic stellate cells are the most discussed and seemingly most important sources of the myofibroblasts in liver injury, other cellular components of the liver have been identified that are an additional source of fibrosis in the liver. These include portal fibroblasts, liver parenchymal cells that undergo epithelial mesenchymal transition. The proportion of each of these cells contributing to the myofibroblast population is albeit dependent on the etiology of the liver fibrosis. In a study performed by Iwaisako et al., it was discovered that in liver fibrosis due to hepatotoxin effect such as CCL4-induced, the myofibroblasts mainly come from hepatic stellate cells. However, in early cholestatic disease, the major contributors are portal myofibroblasts [2, 26].

Inflammatory mediators as well play a significant role in liver fibrosis. One of such is IL-17, which is a pro-inflammatory and pro-fibrogenic cytokine. It activates NF-kb and signal transducers and activators of transcription (STAT3) in Kupffer cells, leading to upregulation of collagen levels. To support this, it has been discovered that mice lacking IL-17A do not develop fibrosis in response to cholestasis or when exposed to fibrotic toxins such as CCL4. In addition, the antifibrotic effect of endocannabinoid CB2 is mediated through inhibition of IL-17 production [2, 27, 28].

IL-22 also plays a role in liver fibrosis. It exerts its effects through STAT3, which include cell proliferation, tissue repair and wound healing. Its receptor is expressed on hepatocytes and hepatic stellate cells. In cirrhosis, the levels of IL-22 circulating in bloodstream are higher than normal. Additionally, its blood levels can point to the extent of cirrhosis as well as mortality rate [2, 29].

TLR4 is present on Kupffer cells, and, more importantly, has been discovered on stellate cells. Its activation by endogenous ligands such as biglycans and heparin sulfate leads to downregulation of bone morphogenic protein (BMP) and activin membrane-bound inhibitor that normally inhibits TGF-beta, which, as was discussed earlier, is the major fibrogenic cytokine in the liver [1].

HISTOPATHOLOGY OF LIVER FIBROSIS

Histopathology is the final court of appeal in the diagnosis of liver fibrosis. Structural changes in the liver accompany the already known physical manifestations of disease. The methods of diagnosis especially liver biopsy have to be reviewed as in recent times fibrosis is no longer thought to be an irreversible process compared to previous times where it was considered so and as a result any crude methods of diagnosis could be employed.

Some issues concerning liver biopsy include the intra-observer and inter-observer errors that could very often occur, the fact that the scoring systems mostly assess structural changes in the liver rather than degree of fibrosis, the invasiveness of the procedure and the problem of biopsy samples being too small to accurately describe the histopathological state of the liver [30, 31].

Currently, the scoring methods for biopsies used in clinical centers are majorly descriptive. They provide information on the grade (from 0 to 4) which evaluates inflammatory state of the liver by the level of lymphocytic piecemeal necrosis, lobular necroinflammation and portal inflammation and stage which reveals the extent of fibrosis in the liver [30, 32].

EXPERIMENTAL ANIMAL MODELS OF LIVER FIBROSIS

There are myriads of experimental models developed by scientists to study various fibrotic processes in tissues, but we will focus on those pertaining to liver fibrosis in this write-up. Although, in humans, hepatic

fibrosis can be caused by various stimuli, such as congenital, metabolic, parasitic, toxins/drugs, vascular or even inflammatory, the molecular mechanisms that underlie fibrosis are basically the same [33].

After an injury to the liver irrespective of the cause, there is a defined series of molecular changes that occurs which is engineered at the cellular and molecular levels [34]. This process is mainly characterized by cellular activation of hepatic stellate cells which acquire a myofibroblast phenotype and are able to express and deposit large quantities of extracellular matrix components within the liver [35, 36, 37]. For temporary insults, the changes observed are transient and resolve of liver fibrosis may be possible. However, in a sustained injury, there is chronic inflammation and accumulation of the extracellular matrix persist, leading to a progressive replacement of normal liver parenchyma by scar tissue.

This study focuses on the experimental models used in studying hepatic fibrosis and carcinoma. For the ease of discussion, these models have been classified into five: Cholestatic models, Genetically modified models, Dietary models, Toxic models, Models of metabolic liver injury.

1. Cholestatic models

Cholestatic liver injury is a major cause of fibrosis and cirrhosis in patients with acute and chronic liver injury. Surgical bile duct ligation (BDL) is the most common model used to induce obstructive cholestatic injury in mice and rats.

This is the procedure involved: under deep anesthesia, a midsection laparotomy is carried out and then, the common extrahepatic bile duct is ligated two times and dissected. Just after about 21 to 28 days, jaundice is developed by the mice and rats and a strong fibrotic reaction originating from the periportal fields [38]. There are different techniques of operation that have been described for special study settings. Special operating procedures allow reconnection or reanastomosis after bile duct ligation [39]. Some other techniques such as partial BDL or microsurgical methods have as well been described [40, 41].

This model allows a fast and reproducible way to inflict cholestatic liver injury. In addition, this model can be used in transgenic mice easily, allowing the investigation of cholestatic injury in many different study designs.

2. Genetically modified mouse models

As research work continues to expand, especially in recent years, many genetically modified mouse models used to study chronic cholestasis and/or autoimmune liver fibrosis have been described. The genes that have been altered in these mice include the transforming growth factor β receptor type IIa (tgfbr2), multi-drug-resistant gene 2 (MDR2), Ae2a,b, Non-obese diabetic (NOD.c3c4), and interleukin 2Rα (il2ra).

- *Multi-drug-resistant gene 2 (MDR2)*

There is MDR2 in mice and MDR3 in humans and these are class III multi-drug-resistant P-glycoproteins. They act as canalicular phospholipid translocators and are indicated in biliary phospholipid (phosphatidylcholine) excretion. In humans, there is an association between mutations in the ATP-binding cassette transporter (ABCB4) gene encoding MDR3 and the loss of canalicular MDR3 protein and/or protein function loss. These mutations being described are associated with a low biliary phospholipid levels, which then result in an elevated biliary cholesterol saturation index. Furthermore, numerous human diseases are connected to mutations of the ABCB4 gene (progressive familial intrahepatic cholestasis, transient neonatal cholestasis, drug-induced liver injury, low phospholipid–associated cholelithiasis syndrome, adult biliary fibrosis intrahepatic cholestasis of pregnancy) [42].

Similarly, an MDR2 (Abcb4) gene knockout in mice leads to a deficiency in phosphatidylcholine excretion into bile. A Low biliary phospholipid levels stimulates nonpurulent inflammatory cholangitis with portal inflammation and ductular proliferation starting immediately after birth and progressing to end-stage disease in the course of 3 to 6 months. The experimental animals develop a phenotype that resembles sclerosing cholangitis with biliary fibrosis and hepatocellular carcinoma [43].

- Transforming growth factor β receptor type IIa (tgfbr2)

Transgenic mice excessively expressing a dominant-negative TGF-β receptor that is restricted to T cells (dnTGFβRII mice) develop an inflammatory biliary ductular disease that is strongly similar to human Primary biliary cirrhosis (PBC). After a spontaneous production of antimitochondrial antibodies (AMAs) that is directed to the exact same mitochondrial autoantigens as in human disease (for instance, the E2 subunits of the branched chain 2-oxo-acid dehydrogenase complex (BCOADC-E2), of the 2-oxo-glutarate dehydrogenase complex (OGDC-E2) and of the pyruvate dehydrogenase complex (PDC-E2), these mice display a lymphocytic infiltration of the liver with periportal inflammation that is similar to the histological transformation in human PBC [44].

- Interleukin 2rα (il2ra)

Another mice model for the human PBC developed is a knockout mouse strain that lacks the gene for interleukin 2 receptor, α chain (IL2Rα). These mice consequentially developed portal inflammation as well as biliary ductular injury that is similar to that of human patients. The portal cell infiltrates show lots of CD4+ and CD8+ T cells and an increased levels of tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), IL-12p40, and IL-2, thus, indicating a type 1 T helper (Th1) cytokine–dominated immune response. These mice do not only develop significantly elevated serum levels of IgG and IgA but also show antimitochondrial antibodies (AMAs) that is specific for PDC-E2, typically seen in human PBC [45].

- Ae2a,b

The expression of AMAs, paired with both pathological and immunological findings that is similar to human PBC, is found also in mice with a defective Ae2a,b gene. Not only an enlarged spleen, increased IL-12p70 production and IFN-γ, a low numbers of CD4+FoxP3+/regulatory T cells and an expanded CD8+ T-cell population, these experimental mice show an extensive portal inflammation with CD8+ and CD4+ T lymphocytes infiltration surrounding the damaged bile ducts. Cholangiocytes were isolated from these mice and they showed gene expression changes that is compatible with oxidative stress and a higher antigen presentation [46].

- Non-obese diabetic (NOD.c3c4)

NOD.c3c4 mice is another model for primary biliary cirrhosis (PBC). They are congenically gotten from the nonobese diabetic strain that develop an autoimmune biliary disease similar to human PBC. B6/B10 regions on chromosomes 3 and 4 containing B6/B10 insulin-dependent diabetes (Idd) loci completely protects these mice for diabetes. Also, they develop AMAs to PDC-E2 that, just as is seen in human PBC, are specific for the inner lipoyl domain. Inflammation of the biliary duct shows infiltration with CD3+, CD4+ and CD8+ T cells. When monoclonal antibodies to CD3 are used to treat NOD.c3c4 mice, they are protected against biliary injury. This model also shows the central role of T cells in developing specific symptoms of PBC. When an adoptive transfer of splenocytes or CD4+ T cells was performed, it was discovered that NOD.c3c4-scid mice develop bile duct injury with the characteristic granuloma formation, destructive cholangitis, and eosinophilic infiltration as also seen in human PBC. However, it was discovered too that the NOD.c3c4 mice also develop extrahepatic biliary ducts injury [47].

3. Dietary models

Dietary models resulting in cholestatic liver injury have been introduced. Examples of these agents include 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or α-naphthylisothiocyanate (ANIT).

- 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC)

Feeding with DDC has been widely used in study of Mallory body formation (as seen in alcoholic liver disease) or oval cell activation and proliferation in murine models of liver damage. Moreover, cholestatic serum markers are significantly induced in these mice. Feeding mice with a diet that has been enriched with 0.1% DDC for 8 weeks results in increased biliary porphyrin secretion. A strong ductular reaction is observed after one week. In epithelial biliary cells, the expression of cytokines as an example vascular cell adhesion

molecule, osteopontin and TNF-α is up regulated. Histopathologically, oral DDC uptake results in pericholangitis with infiltration of inflammatory mononuclear cells and activation of periductal myofibroblasts which cause biliary liver fibrosis that resembles sclerosing cholangitis in humans [48].

- α-*naphthylisothiocyanate (ANIT)*

Another xenobiotic model to induce cholestatic liver injury is feeding mice with ANIT. In general, chronic biliary injury and also increase in the number of bile canaliculi can be induced in mice by feeding them a diet enriched with ANIT in low doses (0.025%), which results in cholestasis several days after feeding [49]. ANIT is conjugated with glutathione in liver cells and is transported into the bile by the Mrp2 transporter [50]. Because glutathione-conjugated ANIT is not stable in bile, it undergoes recycling, absorption and metabolism, leading to bile concentrations that cause direct biliary epithelial cell injury. This injury results in reactive expansion of the biliary epithelium, mild hepatocellular injury and periportal inflammation, and as a result - biliary liver fibrosis [51]. Administration of a single large dose of ANIT (300 mg/kg per body weight) to mice leads to rapid (15 to 24 hours) cholestasis induced by severe destruction of biliary epithelial cells and periportal hepatocellular necrosis [52]. Interestingly, similar intracellular signaling pathways are involved in the mediation of obstructive cholestatic injury (that is, BDL) and ANIT-induced injury. These pathways include the activation of TGF-β and αVβ6 integrins [53, 54].

4. Toxic models

There are several well-established chemical substances that have been identified to induce liver inflammation and fibrogenesis.

The approach most commonly used to initiate a toxin-mediated experimental liver fibrosis is the recurrent introduction of carbon tetrachloride (CCl4) in mice or rats. In mice, about 0.5 to 2-ml/kg-body weight CCl4 (diluted in corn oil) is administered intraperitoneally two to three times a week. This results in a large and highly propagating liver fibrosis between 4 and 6 weeks of treatment. The standard method for the induction of cirrhosis with portal hypertension is the long-term intoxication using inhalation with oral gavage being an alternative application route [55]. However, it was observed 40 years ago that oral CCl4 application is associated with frequent early mortality [56]. The susceptibility to CCl4-induced liver fibrosis in mice depends largely on the genetic foundation. BALB/c exposed mice are most sensitive to fibrosis induction, whereas FVB/N mice have a lower response to CCl4 [57]. Although C57BL/6 inbred mice develop only intermediate liver fibrosis, this strain is frequently used for fibrosis studies in the CCl4 model because of the ready availability of respective knockout mutants or other gene modifications. CCl4 is metabolized by hepatocytes, giving rise to toxic trichloromethyl (CCl3) radicals, which mediate cytotoxic effects which further lead to massive centrilobular liver necrosis [58]. In addition, some evidence have proven that CCl4 may lead to the apoptotic cell death of hepatocytes, although this might be an additional effect and has not been investigated fully up till date [59].

The kinetics of fibrosis development can be roughly divided into three phases: acute injury, initiation of fiber formation and advanced fibrosis. The phase of acute CCl4-mediated liver fibrosis is characterized by activation of liver macrophages called Kupffer cells and the initiation of an inflammatory response. This results in production of cytokines, chemokines and other pro-inflammatory factors. Eventually, this attracts and activates monocytes, neutrophils and lymphocytes, which subsequently leads to liver necrosis followed by a strong regenerative response that results in substantial proliferation of hepatocytes and non-parenchymal liver cells at around 48 hours after the first CCl4 application [60, 61]. Thus, a single CCl4 injection in mice can also be used as a highly procreative model of liver rejuvenation after toxic injury. The first appearance of histological fibrosis and scarring fibers is usually observed after 2 to 3 weeks of CCl4 treatment, depending on the dosage and mouse strains used. Molecular fibrosis markers are also easily detectable at this time. Accordingly, mouse mutants that are expected to display accelerated onset of liver fibrosis can be analyzed after 2 weeks of continuous treatment. True bridging fibrosis can be observed after 4 to 6 weeks of continuous treatment, corresponding to approximately 8 to 18 injections. Of note, CCl4-induced liver fibrosis in mice can be completely resolved within several weeks after withdrawal of the toxic treatment [62, 63]. Thus, the CCl4 model resembles all-important properties of human liver fibrosis, including inflammation, regeneration, fiber formation and potentially fibrosis regression.

Furthermore, another well-established model of experimental liver fibrosis in rodents is the continuous admission of thioacetamide (TAA). Originally established in rats, it is also applied in mice as most times as possible and often serves as a second, independent approach to confirm data obtained from, for example, CCl4-treated animals [64, 65, 66]. Although it is known to be a major inducer of liver injury for decades, the molecular mechanism of TAA-induced liver fibrosis is still not mostly understood. TAA is made biologically active in the liver via oxidation processes, which leads to formation of the by-products, S-oxide and the highly reactive S, S-dioxide. These assuredly are responsible for TAA adverse effect, hepatotoxicity [67]. Previous research suggested that TAA bio-activation involves the hepatic cytochrome P450 enzyme CYP2E2 [68, 69].

TAA can be introduced intraperitoneally at varying concentrations ranging from 150 to 200 mg/kg body weight three times in a week or administered orally by adding 200 mg/L of TAA to the drinking water [70, 71]. Intraperitoneal admission of TAA results in hepatic centrilobular necrosis, elevated transaminase activity and significant liver fibrosis within 6 weeks. Interestingly, when oral TAA was administered, it did not lead to significant elevation of transaminases in mice [72], this further contributed to a lower stress load for animals used in the experiment. In addition, the situation in hepatitis patients with only mild elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) has a striking resemblance to this scenario, but it still has a higher chance of leading to liver fibrosis. However, administering TAA orally requires a more consistent application to cause a similar strength of liver fibrosis compared to the 6-week intraperitoneal treatment with CCl4 or TAA. However, the consequences of oral application of toxins on the gastrointestinal tract, which should be expected was not analyzed in detail in these studies.

Experimental liver fibrosis, although not used as often in the study of fibrosis can also be brought about by the continuous introduction of the hepatocarcinogen, dimethylnitrosamine (DMN) [73]. Its mechanism of action is quite similar to that of diethylnitrosamine (DEN), which is described in detail further below. It has been described that the intraperitoneal injection of 10 mg/kg DMN two times a week gave rise to liver fibrosis within 4 weeks. This was associated with the activation of hepatic stellate cells, Kupffer cells and expression of pro-fibrotic cytokines, hence classifying DMN as a confirmation drug capable of inducing prototypical pro-fibrotic mechanism. However, DMN also has strong mutagenic and carcinogenic properties. Therefore, the analysis made based on the fundamental pro-fibrotic mechanisms in this experimental model could be very complicating because of the overlapping or even mutated signaling pathways [74].

Most studies still rely on the CCl4-model to induce toxic liver fibrosis in mice due to the good comparability with the abundance of previous publications, excellent reproducibility and appropriate stress for the animals. When giving TAA, the application mode should be carefully considered as intraperitoneal as this result in strong injury (similar to CCl4), as oral administration resembles mild hepatitis reflecting, for example, alcoholic liver disease. The DMN model is especially captivating, if the progression from fibrosis to cancer is within the area of interest.

5. Models of metabolic liver injury

The most common chronic liver disease across the globe is the nonalcoholic fatty liver disease (NAFLD) which majority of the times progresses to non-alcoholic steatohepatitis. Non-alcoholic steatohepatitis (NASH) can be described as intralobular inflammation along with ballooning of hepatocytes in contrast with its precursor NAFLD which is simply buildup of fat inclusion within the liver cells. Consequently, fibrotic remodeling of the liver ensues which puts a patient at a high risk of developing hepatocellular carcinoma (HCC). Metabolic syndrome which is generally described as insulin resistance, dyslipidemia, central obesity and glucose intolerance can be pathogenetically identified as a trigger as the characteristics of NASH can be explained as the liver's reaction to the condition of the body [75, 76].

Presently, animal models that mimic the exact pathophysiology and histology of NASH have not been developed. However, some dietary and genetic mouse models that have a fairly similar process have been developed over the decades. In this section, we shall be focusing on one genetic model and three other dietary models of NASH.

The first is a model with a high fat diet; these mice get 60% to 70% of their caloric intake from a preparation with a high fat content that is fed as desired. Due to variations in gender and genetic makeup of

the models, results may vary. C57BL/6J male mice had lesser hepatic lipid accumulation after been fed the high fat diet in contrast to the stronger accumulation in male Balb/C mice [77]. In another case, Wistar rats failed to develop steatohepatitis while Sprague-Dawley rats developed steatohepatitis when both were fed with a high fat diet. Increased plasma insulin level which greatly points to insulin resistance a characteristic of metabolic syndrome, was also discovered on administration of high fat diets. Increased transaminases and signs of hepatic inflammation and fibrosis were noticed in rats after 4 weeks on high-fat diet asides the steatosis and increased lipid content already observed [78,79].

An alcohol containing liquid diet to study alcohol induced liver diseases had been developed about 50 years ago by Lieber and DeCarli [80]. However, if no secondary assaults occur, the only changes occurring would be a mild rise in transaminase levels, very little if any inflammation and mild fat accumulation. Due to these reasons, it was debated if these would be an accurate model for the effect of chronic alcohol consumption as well as the consequent liver pathologies. As a result, the processes have been adjusted in order for researchers investigating alcoholic liver injury to be better equipped [81, 82].

In contrast to high fat models who develop steatosis at a much slower pace, methionine and choline deprived diet (MCD) leads to steatosis as early as the second week into the diet [83, 84]. The MCD diet is made up of 40% sucrose and 10% fat. Methionine and choline play a major role in the synthesis of phosphatidylcholine, methionine and choline have essential functions. And phosphatidyl choline is involved in the secretion of liver triglycerides and subsequent transport as VLDL out of the liver [85, 86]. With an MCD diet, a major enzyme in triglyceride production stearoyl-coenzyme A desaturase 1 (SCD-1), is downregulated [87]. Increase in cytochrome P450 enzymes especially CYP2E1 which signifies oxidative stress, as well as the improvement in steatohepatitis with antioxidant function in addition to the alterations in cytokine and adipocytokine expression, also account for progressive liver injury [88, 89]. Together with reduction in antioxidants such as glutathione, ROS induce oxidative stress and promote steatohepatitis as well as elevated levels of TNF-a. A greater damage to mitochondrial DNA, increased cell death by apoptosis as well as stronger production of ROS is provoked by an MCD in contrast to dietary models, proving it as a highly appropriate model for study of NASH induced inflammation. This however does not imply it is without shortcomings. One of its disadvantages is the variation in amount of liver injury amongst mice and rats as well as different strains. The 8- to 10-week-old female mice from seven different inbred strains (A/J, AKR/J, Balb/cJ, C57BL/6J, DBA/2J, C3H/HeJ and 129X1/SvJwT), were intensively studied for the purpose of comparison, and this revealed that the different mice showed a general difference when fed an MCD diet in regard to ALT, liver weight and liver fibrosis [90]. A more recent study which compared chemokine (C-C motif) ligand 2 (Ccl2)-deficient mice on two different genetic backgrounds (that is, Balb/C and C57BL/6J) discovered related results [91].

In addition, it is also known that males while showing less steatosis develop greater attributes of NASH [92]. The most limiting factor of MCD is the failure of its metabolic profile to accurately show all characteristics of NASH in humans. For example, an MCD diet causes particular weight loss of the animal along with the decreased cholesterol levels as well as plasma triglyceride. Serum insulin, leptin and glucose levels are also decreased with increasing or unchanged levels of adiponectin [88, 93]. Surprisingly, this models displays little or no insulin resistance [94].

Another alternative for NASH induction would be administration of a solely choline deficient (CD) diet. Choline is important for VLDL degradation. The process takes approximately 10 weeks for models to show steatosis, inflammation and fibrosis. However, in a clear contrast to MCD fed mice, these mice have no variations in body weight when compared with the control group [95]. These mice were also insulin-resistant and displayed higher plasma lipids compared to the MCD group, which, had greater steatosis and inflammation [96]. Ethionine was added to the CD diet as a supplement in order to elicit a stronger NASH reaction (referred to as the choline-deficient, ethionine-supplemented.

Ethionine is a methionine antagonist and is usually provided in drinking water. But it also affects hepatocyte proliferation, making it a useful model for the study of hepatic progenitor cells [97].

NASH can also be provoked in animals through genetically altered mouse models. The ob/ob (obese) mouse lacking functional leptin is one of the most common of these type of models used. Leptin is a hormone derived from adipose tissue. These mice become extremely obese, hyperphagic, inactive and insulin-resistant, and they exhibit hyperglycemia together with hyperinsulinemia and eventually develop hepatic steatosis [98].

Thus, within these mice, characteristic metabolic malfunctions clearly reflect NAFLD. Despite this, the changes to steatohepatitis are not spontaneous. It is also critical for extra stimuli such as a high fat diet discussed above or an MCD diet to be administered [99, 100]. Strikingly, these mice do not develop fibrosis even when treated with CCl4 or TAA, which may point to a participation of leptin in fibrotic changes in the liver [93, 101, 102].

NASH development is the result of a myriad of metabolic, inflammatory and structural changes affecting liver physiology and function. Dietary models and genetic modified animals can be used to mimic changes appearing in human NAFLD and NASH, however, none of these disease models accurately shows the disease process in its entirety. As a result, to decide for or against any model should always be based on the emphasis of the study. This shows that it is important for the different models to be properly analyzed alongside each other to prevent experimental failures.

As it is obvious that nothing in life is altogether perfect, more often than not, it is a balance between advantages and disadvantages. For some of the models earlier talked about, the main advantages of this model are technical feasibility, short time to achieve typical disease, reproducibility and high similarity with humans in terms of portal hypertension. One of the limitations in rats and mice is the development of a biliary cyst compressing the portal vein and the stomach. Setting the ligation far within the hilum or injecting Ethibloc or formalin into the bile duct prior ligation prevents this problem [100,101,102].

TREATMENT OF LIVER FIBROSIS

It is well known that cirrhosis is irreversible, but in contrast with the traditional view that fibrosis is also an irreversible condition, recent investigations have showed that fibrosis can be reversible. There is very clear evidence that the most effective prevention of liver fibrosis is the removal of the causative agent. But in many cases this lead-up is not acceptable, and so there is no single standard strategy for treatment of liver fibrosis. Because inflammation has dominant responsibility for the progression of the liver fibrosis, the drug strategies to prevent fibrosis should focus first of all on reducing hepatic inflammation, inhibition of fibrogenic activity of stellate cells and stimulation of the matrix degradation [103].

Corticosteroids are powerful anti-inflammatory drugs that are commonly indicated for the treatment of hepatic fibrosis in patients with autoimmune and alcoholic hepatitis. Considering that chronic liver inflammation, which could be due to alcoholism or hepatic infection, is a preceding factor of fibrosis, anti-inflammatory drugs play a key role in lowering the development of fibrosis [104].

The myeloid cells express inflammasomes (multiproteins that have a few monomer units and are composed of caspases), which are important constituents of the inborn immune system. Inflammasomes are involved in the maturation of the interleukin 1β and 18 (pro-inflammatory cytokines). Recent reports developed pointing toward perspective compounds such as glyburide, ketone body β-hydroxybutyrate, MCC950 that target inflammasome activity in hepatic fibrosis. [105].

Antioxidants showed encouraging results. Vitamin E, phosphatidylcholine, silymarin, and S-adenosyl-L-methionine are capable of stopping hepatic stellate cell activation, shielding hepatocytes from undergoing cell death, hence reducing fibrosis in the liver [106]. These groups of drugs have helpful effects in people with alcohol-induced liver disease via interrupting TGF-beta synthesis and signaling pathways to inhibit the formation of scar [107].

Pentoxifylline (phosphodiester inhibitor), S-farnesylthiosalicylic acid (RAS antagonist) also may have the important ability to treat liver fibrosis by halting signals with major roles in transduction pathways involved in liver fibrosis [108].

In experimental liver fibrosis, thiazolidinediones (peroxisome proliferator-activated receptor ligands) have beneficial results and can have a potentially good result in patients with non-alcoholic steatohepatitis [109].

Animal studies revealed that blockade of endothelin-1 type A receptors by drugs such as bosentan and ambrisentan can exert antifibrotic activity in these animals by blocking collagen synthesis and deposition in these animals, but the effects of blockade on humans are still unknown [110].

Some herbal compounds from traditional Chinese medicine have an inhibitory effect on collagen production and the promotion of its degradation. These compounds are Sho-saiko-to, glycyrrhizin and Salvia miltiorrhiza [111].

Fraxinus Rhynchophylla ethanol extract also showed promising results in an experiment on carbon tetrachloride (CCl4) induced liver fibrosis [112]. Its mechanism of action is based on its ability to downregulate the expression of matrix metalloproteinase-2 (MMP-2), urokinase plasminogen activator (uPA), and tissue inhibitors of matrix metalloproteinase-1 (TIMP-1), all of which influence fibrosis. These factors are important in formation of new vessels and tissue remodeling which can favor tumor cells and are also propagating an increase in the number of hepatic stellate cells as well as their ECM, hence increasing the probability of fibrosis [113].

Nintedanib is another drug known to counteract fibrosis by inhibiting several receptor tyrosine kinases, including vascular endothelial growth factor receptors, platelet-derived growth factor receptors, and fibroblast growth factor receptors (VEGFR, PDGFR, FGFR, respectively). It also ceases the action of Fms-like tyrosine kinase (FLT), and non-receptor tyrosine kinases Lck, Lyn and Src [114].

According to recent animal studies, platelets have the ability to directly activate stellate cells hence they induce liver fibrosis. So in case of platelet inhibition we might be able to decrease progression of the liver fibrosis. Despite reversibility of liver fibrosis by platelet inhibition still have not been proved in human studies, administration of aspirin was associated with a decrease in composite liver fibrosis index [115]. Therefore, antiplatelet drugs have good potential for further investigation of their effect on the prevention and treatment of liver fibrosis.

Apart from aspirin, any drugs with the ability to inhibit nitric oxide synthase may have the potential to prevent liver fibrosis in high cholesterol diet-induced cases. The idea in this case is that high cholesterol diet can eventually cause liver fibrosis (accumulation of cholesterol in the liver is associated with the development of non-alcoholic steatohepatitis related fibrosis). The pro-fibrotic role of nitric oxide synthase (iNOS) has been demonstrated on mice. So the usage of iNOS inhibitors can potentially aid in prevention of liver fibrosis in patients with high cholesterol level [116].

In several recent studies, investigators tried to link coffee consumption with the severity of nonalcoholic fatty liver disease, and an association had been established between consumption of coffee and a decrease in risk of liver disease progression. Coffee intake can lower the liver enzymes level in the blood, which can lead to the reduction of mortality risk for patients with liver cirrhosis. Also, coffee consumption is linked to decrease in clinical and pathologic progression of liver fibrosis in patients with chronic hepatitis C [117].

Another investigator established a study in which the rats receiving high cholesterol diet were given decaffeinated coffee and had lower levels of hepatic fat and collagen and less liver inflammation, compared to rats in control group. This study emphasizes the importance of coffee itself rather than just caffeine in preventing of liver fibrosis [118].

Non-alcoholic fatty liver disease patients who take statins have reduction in risk of obtaining non-alcoholic steatohepatitis and fibrosis. In contrast, insulin is associated with greater risk of non-alcoholic steatohepatitis and subsequent fibrosis development [119]. But it is worth mentioning that although statins do not induce real hepatotoxicity, they can increase liver function test values.

Transforming growth factor beta (TGF-beta) is an essential chemotactic factor for fibroblasts, and stimulation of fibroblast proliferation leads to the increase in synthesis of extracellular matrix and collagen. In case of injury, TGF-beta 1 activity is considered a normal reaction and helps in tissue repair and healing process, but persistence of its activity can lead to tissue scarring and fibrosis. Phosphorothioate decoy or other decoys decrease procollagen gene expression, hence decreasing collagen synthesis during fibrogenesis. The main idea is that these decoys act as promoter competitors and bind to activator proteins either in nucleus or cytoplasm that block the effects of TGF-beta 1 on the collagen synthesis [120].

GM-CT-01 and GR-MD-02 – drugs that bind galectin-3 (a carbohydrate binding protein in human body which is a member of lectin family) and can be used in non-alcoholic steatohepatitis with fibrosis. They are involved in a significant reduction of collagen deposition. GR-MD-02 also reduces the expression of pathological indicators such as iNOS and alpha smooth muscle actin, with latter being a marker for activation of stellate cells [121]. However, mechanism of action of these two new compounds requires more investigation.

Another perspective concept can be inhibition of the pan-caspases, which will also lead to a reduction in the severity of fibrosis. Phagocytosis of apoptotic cells activates stellate cells, hence inhibiting hepatic apoptosis, which suppresses the development of fibrosis. Pan-caspase inhibitor VX 166 would reduce progression of fibrosis and can be a perspective drug for treatment of fibrosis [122].

Doxorubicin (DOX) inhibits liver fibrosis by conjugation to stellate cell-selective carrier called manose-6 phosphate-modified human serum albumin [m6PHSA]. The resulting m6PHSA-DOX complex specifically enters the stellate cells and strongly attenuates stellate cell proliferation in vitro [123].

Pirfenidone inhibits proliferation; arrests cell cycle and down regulates heat shock protein-47 and collagen type 1 in rat hepatic stellate cells in vitro. Overexpression of heat shock protein-47 enhances TGF-beta 1 activity, and some studies suggest that heat shock protein-47 plays a major rule in hepatic fibrosis by inducing expression of collagen type 1 in stellate cells [124].

Another alternative way for prevention collagen synthesis can be modification of phosphatidylinositol3-kinase (PI3K) signaling pathway in hepatic stellate cells. Some investigations showed that inhibition of PI3K during active fibrogenesis decreases extracellular matrix deposition, collagen1 synthesis and expression of profibrotic factors [125].

CONCLUSION

The primary aim of this review article is to increase awareness of healthcare professionals, researchers and general public regarding liver fibrosis by providing information on etiology, pathogenesis, histopathology, and available and experimental treatment options for liver fibrosis; extensive description of current experimental models used for research of liver fibrosis is also present. Future opportunities for research on liver fibrosis and development of effective treatment strategies are promising, and implementation of new therapies would be beneficial for health and quality of life for currently affected individuals, as well as those at risk groups.

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