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MINI REVIEW ARTICLE

Neovascularization is a key feature of liver fibrosis progression: anti-angiogenesis as an innovative way of liver fibrosis treatment

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Abstract

Liver fibrosis affects over 100 million people in the world; it represents a multifactorial, fibro-inflammatory disorder characterized by exacerbated production of extracellular matrix with consequent aberration of hepatic tissue. The aetiology of this disease is very complex and seems to involve a broad spectrum of factors including the lifestyle, environment factors, genes and epigenetic changes. More evidences indicate that angiogenesis, a process consisting in the formation of new blood vessels from pre-existing vessels, plays a crucial role in the progression of liver fibrosis. Central to the pathogenesis of liver fibrosis is the hepatic stellate cells (HSCs) which represent a crossroad among inflammation, fibrosis and angiogenesis. Quiescent HSCs can be stimulated by a host of growth factors, pro-inflammatory mediators produced by damaged resident liver cell types, as well as by hypoxia, contributing to neoangiogenesis, which in turn can be a bridge between acute and chronic inflammation. As matter of fact, studies demonstrated that neutralization of vascular endothelial growth factor as well as other proangiogenic agents can attenuate the progression of liver fibrosis. With this review, our intent is to discuss the cause and the role of angiogenesis in liver fibrosis focusing on the current knowledge about the impact of anti-angiogenetic therapies in this pathology.

Keywords Liver fibrosis · Angiogenesis · Anti-angiogenic drugs · Liver information · Liver fibrosis regression

Introduction

Genetic, environmental and lifestyle factors (e.g. alcohol abuse), mechanotransduction signal pathway and viral infections can contribute in onset and progression of liver fibrosis (LF) [1–6]. Histologically, this disorder can be classified as a chronic fibro-inflammatory condition characterized by an excessive deposition of extracellular matrix (ECM) proteins including collagen fibers (I, III, and IV) [7–9]. Clinically, portal hypertension can be a key feature in patients suffering from severe form of LF [10, 11]. Evidence from a number of studies demonstrates that angiogenesis, the formation of new blood vessels from pre-existing vasculature, plays a crucial role in the progression of this complex disease [12–15]. It is well known that inflammation and hypoxia are two elements that strongly promote neovascularization [16–18]. Interestingly, both phenomena can be considered as markers of LF [19, 20]; thus, it is reasonable that angiogenesis takes place during hepatic fibrogenesis [12, 13]. Consequently, it is conceivable that anti-angiogenic approaches could represent a useful tool in the treatment of LF. The present review will describe the general aspects of the pathogenesis of LF, focusing on the link between hepatic fibrogenesis and angiogenesis. Meanwhile, selective strategies targeting angiogenesis for the preservation of the hepatic tissue will be introduced.

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The pathogenesis of liver fibrosis

As aforementioned, histologically, fibrotic hepatic parenchyma is characterised by chronic inflammation and exacerbated production of ECM molecules with consequent abnormality in the liver tissue [7, 8]. The inflammatory foci are comprised of lymphocytes, plasma cells, monocytes/macrophages (LY6C^{hi} phenotype) as well as granulocytes [21, 22]. All these inflammatory components indirectly participate in the process of fibrogenesis by producing soluble/paracrine signals including cytokines, chemotactic molecules, fibrogenic agents [23, 24]. Also, in the chronic hepatic injuries, cholangiocytes, hepatocytes, liver sinusoidal endothelial cells (LSECs) and non-sinusoidal endothelial cells (ECs), together with resident Kupffer cells secrete various sclerotic stimuli such as transforming growth factor- β (TGF-β, the "master mediator" of many fibrotic disorders), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) [25]. Figure 1 depicts the many cell types and molecular effectors involved in LF, leading to the activation of HSCs. Generally, the primary effectors of fibrogenesis are resident fibroblasts, myofibroblasts [26, 27], and their bone marrow-derived circulating precursors namely fibrocytes [28, 29]. In damaged liver, activated hepatic stellate cells (HSCs) are mainly

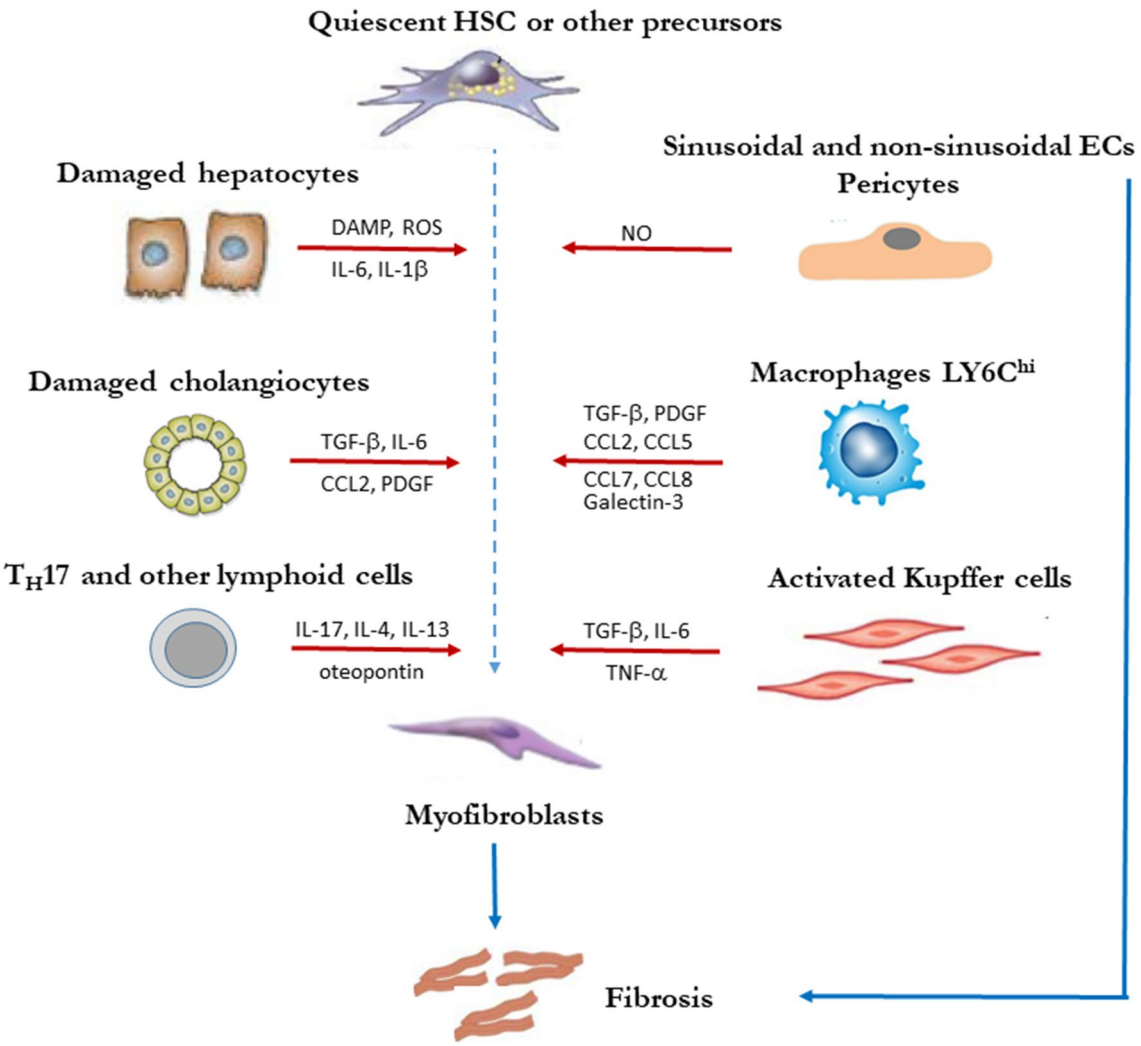

Fig. 1 During liver injury, quiescent HSCs or other precursors (e.g. bone marrow-derived fibrocytes, portal fibroblasts, hepatocytes in the epithelial-mesenchymal transition) are activated by various cell types resident in the liver, including hepatocytes, cholangiocytes, sinusoidal and non-sinusoidal endothelial cells, pericytes, macrophages LY6C^{hi}, Kupffer cells, as well as Th17 T cells and other lymphoid cells. All these cell types secrete pro-fibrogenic mediators that ultimately activate HSCs or other precursors that eventually transform into myofibroblasts and operate to deposit ECM. *CCL* CC chemokine ligands, *DAMP* danger associated molecular pattern, *IL* interleukin, *NO* nitric oxide, *PDGF* platelet-derived growth factor, *ROS* reactive oxygen species, *TGF-β* transforming growth factor-β, *TNF-α* tumor necrosis factor-α

responsible of fibrogenesis in at least 2 ways: on one hand they produce ECM, on the other hand impede ECM degradation by secreting proteases inhibitors including endogenous tissue inhibitors of metalloproteinases (TIMPs) [30, 31]. There is a lot of evidence showing that epithelial-mesenchymal-transition (EMT) has also a great importance in fibrotic lesions. Accordingly, hepatocytes as well as ECs/LSECs can undergo a process of epithelial-(endothelial)-mesenchymal transition (EMT) through autocrine/paracrine signals mediated, in part, by TGF-β [32]. Additionally, recent work discovered that pericytes, are also involved in the process of fibrogenesis [33–35]. In fact, these mural cells seem to have the capability to detach from basement membrane surrounding hepatic capillary to accumulate within the injured hepatic tissues, where they undergo phenotypic transformation into ECM-producing myofibroblasts [33–35]. As a consequence, a wide range of cells, growth factors and other stimuli are engaged in the liver fibrogenesis [33–35].

The link between angiogenesis and liver fibrosis

Angiogenesis is a growth factor-dependent phenomenon taking place during all stage of the human development; during adult life, at least in healthy conditions, it happens only in certain circumstances, for example during pregnancy and menstrual cycle [36, 37]. By contrast, experimental and clinical evidences indicate that angiogenesis accelerates the progression of many disorders such as cancer growth and metastasis, rheumatoid arthritis, diabetic retinopathy and other complex diseases including LF [12, 38–42]. It is well known that inflammation and hypoxia are crucial elements in induction of neovascularization. As previously specified, hepatic tissue affected by fibrosis, shows permanent inflammation and low oxygen level, offering a prototypical microenvironment for neovascularization [43, 44]. In detail, accumulation of ECM in liver parenchyma is a main cause of hypoxia, which in turn, stabilizes the dimeric transcription factor "hypoxia-inducible factor" (HIF) [45]. HIF regulates the transcription of an array of genes including those controlling angiogenesis such VEGF, PDGF-B, matrix metalloproteinases (MMPs) as well as TIMPs [46–49]. As matter of fact, hypoxic areas co-localize with those of an increased microvessel density (MVD), fibrous septa and inflammatory foci [48, 50, 51]. In addition, hypoxia further stimulates the infiltration of inflammatory cells [52], which, in turn, contribute to angiogenesis and fibrotic phenomena [53]. In conclusion, in injured liver, hypoxia, angiogenesis, chronic inflammation and fibrosis drive each other following an activated loop, and synergistically exacerbate the severity of the LF (Fig. 2) [15, 16, 54].

To incite neoangiogenesis, VEGF binds to its receptors VEGFRs stimulating the formation of new functional vessels (Fig. 3). By the signals generated by bound VEGFRs, VEGF is the leading regulator of ECs/LSECs activity during all steps of angiogenesis [55]. In LF, VEGF is, in part, produced by ECs/LSECs themselves suggesting an autocrine action of this signal pathway; but damaged hepatocytes and activated HSCs seem to be the principal sources of this relevant growth factor [56]. The latter evidence highlights the crucial role of HSCs in LF because they constitute a crossroad among inflammation, fibrosis and angiogenesis (Fig. 4) [12, 16, 57, 58]. PDGF-B, principally produced by ECs/LSECs, acts during vessel stabilization, orchestrating the formation/maturation of vascular tube and its coverage through the recruitment of PDGFRs

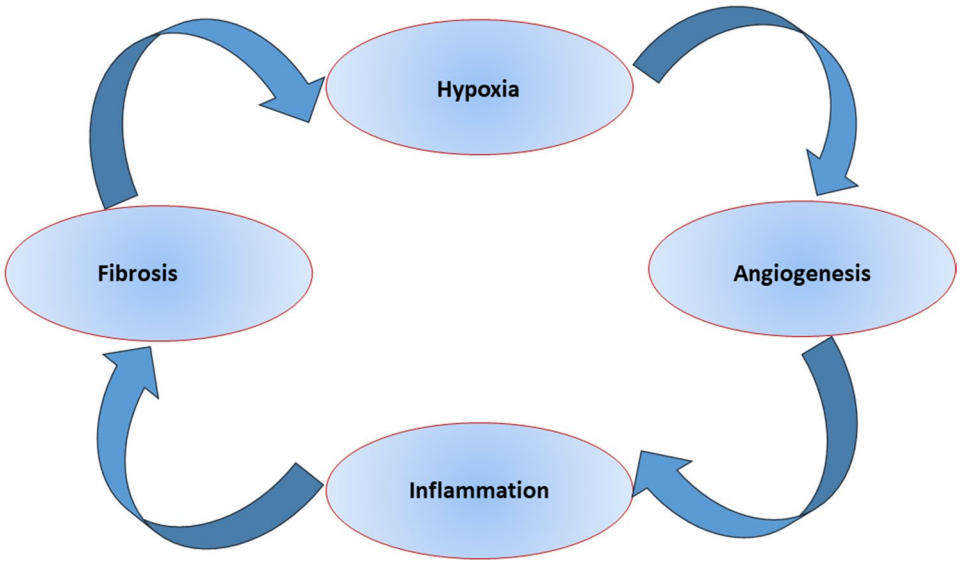

Fig. 2 Link among hypoxia, angiogenesis, inflammation and liver fibrosis. Hypoxia, angiogenesis, inflammation and fibrosis drive each other activating a pathological loop in liver

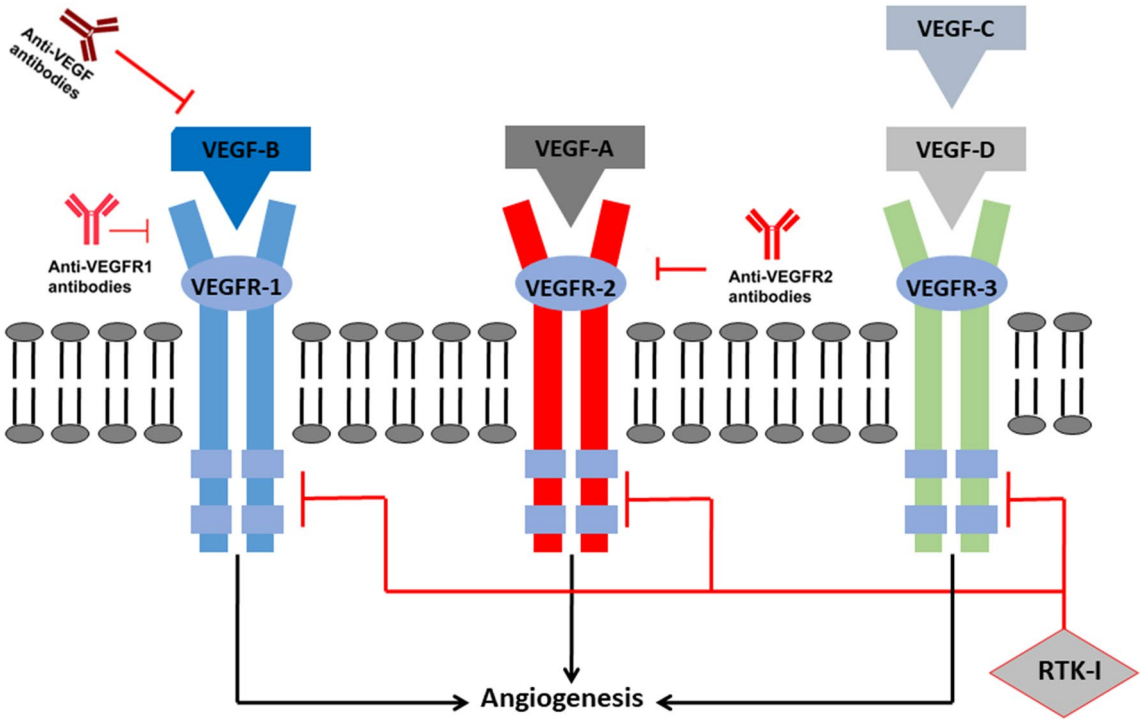

Fig. 3 The VEGF/VEGFR signalling axis, its contribution to angiogenesis and treatment modalities interfering with its activity. Binding of VEGF ligands to their cognate receptors (VEGFRs) leads to receptor dimerization and autophosphorylation triggering a downstream intracellular phosphorylation cascade. Monoclonal antibodies target VEGFs, preventing its binding to VEGFRs, while monoclonal antibodies targeting VEGFRs prevent the binding of VEGFs, resulting in the inhibition of VEGFR signalling. The treatment of receptor tyrosine kinase inhibitors (RTK-Is) inhibits the activation of VEGF/VEGFR signalling

positive pericytes/HSCs [57]. FGF, through an autocrine loop, is involved in LF angiogenesis not only inducing the activation of ECs/LSECs, but also, increasing HSC proliferation and recruitment [12]. Ang-2 is acutely released from activated ECs/LSECs, upon stimulation with inflammatory cytokines, proangiogenic factors, and hypoxia and competitively inhibits the binding of Ang-1 to Tie-2 [59] that, instead, serves to maintain survival and quiescence of endothelium [60]. Inflammatory cells also secrete a plethora of angiogenic factors (VEGF, PIGF, PDGF, FGF, Angiopoietins, TGF-β, etc.) [12, 61–67]. For example, both infiltrating macrophages and resident Kupffer cells, once activated, contribute to angiogenesis releasing reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis factor- α (TNF-α) and other angiocrine molecules [12]. As above cited, in drastic circumstances, LF is complicated by portal hypertension (PHT) accompanied by severe hepatic structural disorder correlated to diffuse fibrosis [68]. Angiogenesis also participates in the pathogenesis of PHT, in part modulating HSCs activation and, on the other hand, provoking the formation of portal-veins collaterals [69]. Cellular molecules involved in promoting angiogenesis and their roles in LF are listed in Table 1 [57, 70–75].

Anti-angiogenesis approaches slow down LF

Considering the indisputable importance of neovascularization in LF progression, it is plausible that blocking angiogenesis may offer a method to attenuate the aberration of hepatic tissue or prevent more serious damage including cirrhosis [14, 76]. For this reason, some anti-angiogenic strategies including natural compounds are currently under investigation [77]. Since VEGF is the most efficient proangiogenic factor, generally most anti-angiogenic therapies have been focused on blocking the VEGF signal pathway [76, 78] (Fig. 3). Bevacizumab, a humanized monoclonal antibody neutralizing VEGF-A [72, 79, 80], in combination to other drugs, is currently used to treat different kinds of tumors [74, 81, 82]. It also shows a strong anti-fibrotic effect in human Tenon's fibrosis [83]. High VEGF-A levels in the aqueous humor of patients with nonneovascular glaucoma have been reported [84], and this increase may contribute to post-operative inflammation and fibrosis. Since Tenon's fibroblasts have been shown to express VEGF-A receptors [84], these findings highlight once more the intimate relationship between angiogenesis and fibrosis, as it occurs in LF. An interesting study conducted by Huang et al. [85], showed that bevacizumab alleviates LF in vivo by

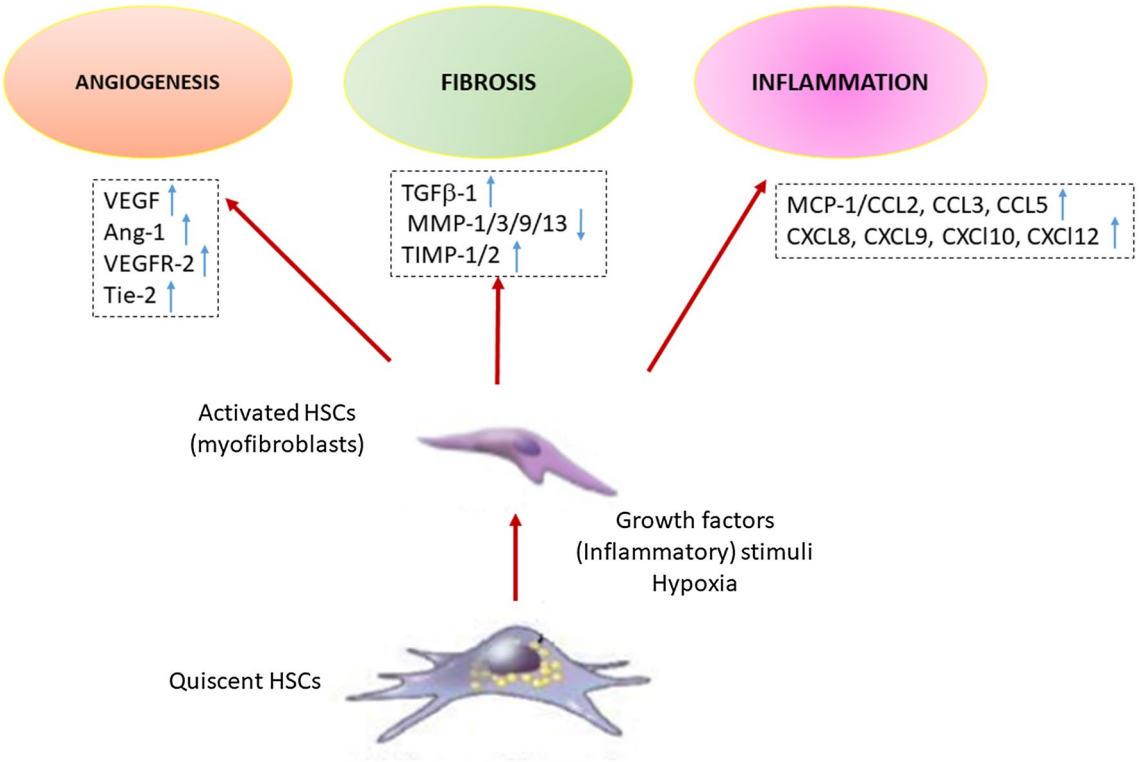

Fig. 4 Schematic model of HSCs activation. Quiescent HSCs are activated, during lung injury, by a host of factors, including hypoxia, inflammatory stimuli and growth factors produced by liver cells, such as hepatocytes and endothelial cells. HSCs transform into myofibroblasts and contribute to angiogenesis, fibrosis and inflammation. Once activated, HSCs act as proangiogenic cells and may respond to stimuli such as hypoxia through the increase of VEGF, Ang-1, and their related receptors VEGFR-2 and Tie-2. Activated HSCs are the prime downstream effectors of excess ECM deposition and they also produce the fibrogenic cytokine TGF-β. Moreover, fibrolysis is compromised, e.g. by an increased synthesis of TIMPs and a decreased production of fibrolytic MMPs. Finally, activated HSCs contribute to inflammation in liver fibrosis by producing chemokines, including CC chemokines ligands (CCL2, CCL3, CCL5) and the CXC chemokines ligands (CXCL8, CXCl9, CXCL10, CXCL12). *MCP-1* monocyte chemoattractant protein-1

neutralizing VEGF produced by hepatocytes and by blocking HSCs activation. The effects of VEGFRs neutralizing antibodies such as anti-VEGFR1 and anti-VEGFR2 have also been explored [86, 87] (Fig. 3). Results show that the use of anti-VEGFR-2 antibody results more effective than the anti-VEGFR-1 antibody when used alone [87], although combined treatment with both antibodies gave some tissue improvement in LF [87]. Additionally, the use of the multiple receptor tyrosine kinase inhibitors such as sorafenib and sunitinib blocking PDGFR-β and VEGFRs signaling pathways is under investigation [88] (Fig. 3). Sorafenib attenuates LF by reducing HSCs proliferation/activation and inducing their apoptosis both in vitro and in vivo [88, 89]. Sunitinib also decreases LF, switching off inflammation, HSCs activation and angiogenesis [90, 91]. PDGF-B and its signaling pathway and cyclooxygenase-2 are also involved in HSCs activation [92, 93]. Gao at al demonstrated that the use of celecoxib (a cyclooxygenase-2 inhibitor) shows similar effect in vivo as those obtained with sorafenib [94]. It is implicit that enhancing the expression and the activation of the ECM proteases can also contribute to the resolution of fibrosis [95, 96]. In line with this idea, it has been shown that the decrease of LF is associated with increased expression of MMPs (MMP-2 and -14) as well as decreased expression of TIMP-1 and -2 in hepatic tissue [30, 97].

Along with the use of immunotarget therapy above listed, recently, the regenerative potential of stem cells is being exploited in fibrotic diseases. Accordingly, the injection of bone marrow-derived mesenchymal stem cells (BMSCs) including endothelial progenitor's cells (EPCs) seems to reduce the severity of LF by increasing the degradation of ECM by means of proteases/MMPs [98, 99]. In fact, experimental evidences showed that EPCs transplantation was shown to effectively promote the remodelling of damaged liver tissues in a dimethylnitrosamine (DMN) rat liver fibrosis model [100, 101].

Other approaches to slow down LF

Additionally, studies have demonstrated that LF may be prevented or reversed by bioactive food components and natural products, including fumagillin analogue (TNP-470),

Table 1 Molecules involved in angiogenesis and their role in LF cited in this review

Angiogenic factor	Actions during angiogenesis	Role in angiogenesis in LF	References
VEGF	• Promotes endothelial cell survival and homeostasis • Promotes endothelial cell detachment from the basement membrane • VEGF and Notch co-operate in an integrated intercellular feedback that functions as a ''branching pattern generator''	Produced by damaged hepatocytes and activated HSC → capillarization of sinusoids	[70]
PDGF-B	Recruitment of pericytes	Produced by ECs/LSECs this factor stimulates HSC proliferation, differentiation, and migration, as well as transforms HSC into myofibroblasts	[57]
TGF-β	Stimulates mural cell induction, differentiation, proliferation, and migration and promotes production of extracellular matrix	Release of TGF-β by necrotic hepatocytes during liver damage is one of the first signals to activate adjacent quiescent HSC → trans-differentiation into myofibroblasts	[71]
FGF	This factor is mitogenic for endothelial cells and increases the expression of VEGF	Induces the activation of ECs/LSECs, and increases HSC proliferation and recruitment	[72, 73]
ANG1 and Tie-2	ANG1, produced by mural cells, activates its endothelial receptor Tie-2 ANG1 stabilizes vessels, promotes pericyte adhesion, and makes them leak resistant by tightening endothelial junctions	Autocrine ANG1 promotes HSC/myofibroblast migration	[70]
EGF and TGF-α	They are mitogenic for endothelial cells and increase angiogenesis in in vivo model	Hepatocyte-derived EGF induces HSC migration Autocrine TGF-α is involved in transformation into myofibroblasts	[74, 75]

astaxanthin, curcumin, blueberry, silymarin, vitamins (C, D, E), resveratrol, quercetin, coffee and green tea extracts [102–104]. Generally, the anti-fibrotic effect of all these natural compounds seems to be mainly attributed not only to their antioxidant and anti-inflammatory features but also to their ability to revert the activated forms of HSCs in a more quiescent phenotype [102].

Current challenges and future directions

Inflammation, fibrosis and angiogenesis are strictly intertwined during the progression of chronic liver diseases (CLDs), including chronic viral hepatitis, PTH, non-alcoholic and alcoholic liver diseases. This brings to the notion that a wealth of cellular and molecular mechanisms are implicated in liver fibrosis and angiogenesis. Interactions among hepatocytes, HSCs, Kupffer cells, and endothelial cells have been described, with HSCs representing a crossroad at the interaction between inflammation, angiogenesis, and fibrosis. Angiogenic factors, including VEGF, PDGF, FGF, Ang-2, EGF, and various cytokines, are important mediators of angiogenesis in fibrosis associated with CLDs. Besides these factors, metabolic abnormalities, including adipokines, may dysregulate angiogenesis, and hence influence inflammation and fibrosis. Moreover, it has also been shown that endoplasmic reticulum stress and related unfolded protein response, and neuropilins are involved in liver angiogenesis and fibrosis [12]. Given the plethora of cellular and molecular mechanisms, a better appraisal of this complexity may be caught by three-dimensional (3D) models that can recapitulate liver architecture and interactions among different cell types [105, 106]. Indeed, one obstacle in the development of efficient therapies is the lack of robust and representative in vitro models of human liver fibrosis through which novel drugs can be tested. Currently used animal models are not useful for dissecting the relative role of each component since the predictive value for human physiologic responses in terms of pharmacokinetics and pharmacodynamics is sometimes poor. Moreover, they are not suitable for large scale screening of antifibrotic compounds. The main 3D models that are being used and implemented include cocultures of hepatocytes and HSCs, achieved by insert cultures, spheroids (presenting many cell types), or liver tissue cultures. More advanced techniques are bioprinting and microfabricated microfluidic devices to provide a constant flow of oxygen and fresh nutrients and remove the metabolic waste generated (as replacement of bile canaliculi). Finally, organotypic models, such as precision cut liver slices and decellularised 3D scaffolds, will offer more opportunities to test novel drugs in a context maintaining the intact hepatic architecture and cellular heterogeneity. Thus far, the main focus of the field has been on the maintenance of functional hepatocytes for prolonged culture periods;

incorporation of non-parenchymal cells (such as endothelial cells, Kupffer cells, and HSCs) will allow the use of these culture systems for in vitro fibrosis studies [105]. In order to gain higher number of cells and make sustainable these models, stem cells are a suitable source of different cell types. Many stem cell types, including liver progenitor/stem cells, extra-hepatic biliary tree stem cells, embryonic stem cells as well as induced pluripotent stem cells (iPSCs) have been reported to generate hepatocyte-like cells [107] and cholangiocytes [108, 109], and more recently LSECs and HSCs [110]. Further studies should determine whether these cell types are fully functional and can reconstitute organotypic models. Another essential feature of these models will be the inclusion of stiffer materials mimicking the deposition of collagen that is a feature of liver fibrosis. Depending on the hardness of the substrates used, i.e. soft versus stiff, the quiescent phenotype of HSCs will be maintained, or they will transform into activated myofibroblasts [5, 106]. Recently, a novel 3D organotypic liver models comprised of hepatocytes, LSECs, HSCs, Kupffer cells, and the Space of Disse mimic demonstrated how a mechanical gradient resulted in transitioning phenotypes in hepatic cells and cause varying profiles of fibrotic markers [111]. Thus, mechanotransduction and biomechanics are parameters that should be envisioned as essential in constructing these models. These advanced in vitro models have been used for testing drug induced liver injury, determined by alcohol or medications, in the developmental phase of pharmaceuticals [105, 112] or in the evaluation of drugs already in clinical trials [113]. In addition to inhibitors of angiogenesis, that could result in unspecific effects, genetic tools may target profibrotic and proangiogenetic genes with an unprecedented precision. Small interfering RNAs and antisense oligonucleotides have been vehicled by nanocarriers (lipoplexes and nanoparticles) that are preferentially engulfed by nonparenchymal cells, prominently HSCs and myofibroblasts. The target genes to be downregulated include TGFβ-1, TGFβ receptors, osteopontin, integrins, and chemokine receptors [58]. Complex 3D and organotypic models are also essential in finding novel noninvasive markers of angiogenesis in liver fibrosis. Histological follow-up does not have the power to reliably detect antifibrotic drug effects in the short term. Validated serum markers would measure the activity of angiogenesis and fibrogenesis and therefore enable the selection of patients likely to respond to antiangiogenic and antifibrotic therapies, and to detect responders to these therapies. Finally, these models could capture the inter-individual genetic and environmental variations, increasing the pace towards the personalised medicine approach [58], and will be paramount to design more precise and real-to-life clinical trials.

Relevant conclusion

Anti-angiogenic therapy for hepatic fibrosis resolution has received increasing attention in recent years. However, it is not possible to overlook the fact that the LF is a multifactorial disorder, and angiogenesis in only one of the phenomena that favours its genesis and progression. Moreover, the limited preclinical/clinical studies impede to know in detail any counterproductive effects of antiangiogenic therapies in this aberrant circumstance. Consequently, further large randomized studies need to be conducted before deducing that anti-angiogenic approaches can be used in the treatment of liver fibrosis.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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