Angiogenesis in the progression of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide, and an increasing cause of liver cirrhosis and hepatocellular carcinoma. Angiogenesis, the formation of new blood vessels from pre-existing ones, is a key pathophysiological mechanism contributing to NAFLD progression. Major triggers for angiogenesis in NAFLD include tissue hypoxia, structural and dynamic endothelial cell dysfunction, stellate cell activation and macrophage-mediated inflammation. In turn, angiogenesis drives inflammation and is closely linked to the progression of liver fibrosis and the development of liver cancer. In particular, the molecular crosstalk between pro-angiogenic endothelial cells and activated stellate cells can result in a positive feedback loop in which angiogenesis and fibrosis develop in parallel. In this review, we highlight the molecular mechanisms, drivers and consequences of angiogenesis in the progression of NAFLD to NASH, fibrosis and hepatocellular carcinoma. Evidence from animal and clinical studies suggests that mediators of angiogenesis and endothelial dysfunction are promising disease biomarkers, and that inhibiting angiogenesis may improve the course of NAFLD. (Acta gastroenterol. belg., **2020**, 83, **301-307**).

Keywords : NASH, HCC, angiopoietin-2, endothelial dysfunction, stellate cell.

Introduction

As a result of the obesity pandemic, non-alcoholic fatty liver disease (NAFLD) has become the most common cause of chronic liver disease worldwide, both in the adult as well as pediatric population (1-3). NAFLD, particularly its inflammatory form non-alcoholic steatohepatitis (NASH), can progress to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (2). The prevalence of NAFLD is anticipated to increase further over the next 15 years, with a higher proportion of patients progressing to more advanced liver fibrosis, the main histological determinant of both liver-related and overall mortality (4,5). Nevertheless, approved pharmacological therapy is lacking (6), whereas lifestyle modification is difficult to achieve and sustain. Therefore, acquiring an understanding of the underlying mechanisms of disease and how to modulate these is critical.

Blood vessels have co-evolved 600 million years ago to supply organs and tissues with both oxygen and nutrients for energy production (7). Although indispensable, abnormal blood vessels can fuel inflammatory, fibrotic and malignant disease, whereas insufficient vessel growth and perfusion cause ischemic disease (8). Angiogenesis, a complex, dynamic and growth factor-dependent process leading to the formation of new blood vessels from preexisting ones, is strongly associated with scar formation and sinusoidal remodeling in chronic liver diseases (9). Although angiogenesis and fibrogenesis are interconnected events irrespective of liver disease etiology, the mechanisms, mediators and consequences are in part context- and injury-specific.

This review synthesizes recent studies on angiogenesis in NAFLD, focusing on the crosstalk with fibrogenesis.

Modes and mediators of angiogenesis

Different modes of vessel formation have been identified, some of which are exclusive to developing tumors. An extensive overview of all modes and mechanisms of vessel formation (reviewed in (8,10)) is outside the scope of this article. Briefly, angiogenesis is distinct from vasculogenesis, a process mainly responsible for *de novo* vessel formation in the developing embryo. Vasculogenesis is uncommon in adults, but can occur in pathological circumstances through the differentiation of circulating endothelial progenitor cells (8). Intussusception refers to the splitting of an existing vessel in two by the formation of a tissue pillar (11).

Sprouting angiogenesis is the classical form of angiogenesis and occurs in a well-characterized sequence. Firstly, destabilizing factors, such as Angiopoietin-2 (Ang-2) cause vasodilation, loosening of intercellular junctions, pericyte detachment and extracellular matrix degradation (12). To coordinate the elongation of a vascular tube, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and other angiogenic factors induce the formation of a migrating tip cell, along which neighboring stalk cells proliferate and migrate (8). The direction of the tip cell migration is determined by guidance signals such as ephrins and semaphorins. This process can be inhibited at various steps by thrombospodin-1, endostatin and angiostatin (12). After the new vessel is formed, a phase of arteriogenesis follows, during which pericytes are

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recruited by signals inluding Notch and Ang-1. Finally, endothelial cells resume their tight junction bonds and a new basement membrane is formed (10).

Turning on the angiogenic switch in NAFLD

Multiple interrelated factors trigger angiogenesis in NAFLD, including tissue hypoxia, endothelial dysfunction, hepatic stellate cell (HSC) activation and inflammation.

Because of the unique vascular anatomy of the liver, the perivenous (zone 3) region, where the histological features of NAFLD are typically most prominent, is most susceptible to hypoxia (13). Liver steatosis also exacerbates this hypoxia. Mechanistically, the metabolism of fatty acids increases oxygen consumption and puts high bio-energetic demands on mitochondria, inducing mitochondrial and oxidative damage. Steatosis and fibrosis also impede the oxygen supply by mechanical compression of the sinusoids and by HSC contraction (12,14). Interestingly, patients with obstructive sleep apnea syndrome, which causes intermittent hypoxia, are more susceptible to NAFLD, independent of other risk factors (15). In response to hypoxia, hypoxia-inducible factor (HIF) transcription factors translocate to the nucleus and stimulate angiogenesis (16).

The liver sinusoids are lined by distinct endothelial cells (LSECs) that lack a basement membrane and carry *fenestrae*, which regulate the transport of macromolecules, including lipids, across the sinusoids (17). Endothelial capillarization and dysfunction are early pathophysiological events in NAFLD. In an animal study, capillarization, which denotes the disappearance of *fenestrae* and the emergence of a basement membrane, occurred at the stage of NAFL, before ballooning and inflammatory foci were histologically evident. Recovery with a standard diet led to the disappearance of the histological hallmarks of NAFLD, yet the number of endothelial fenestrations remained significantly lower than in healthy controls (18). These endothelial cells also become dysfunctional, i.e. the generation of vasodilating agents, such as nitric oxide (NO), in response to shear stress is impaired (19). This phenomenon is systemic in nature in NAFLD. For instance, the ischemia-induced vasodilation of the brachial artery was significantly impaired in patients with NASH, and to a lesser extent in patients with isolated steatosis (NAFL) (20).

Dysfunctional endothelial cells secrete pro-angiogenic factors, but also produce pro-fibrogenic factors (such as transforming growth factor (TGF)-β) and lose their NO-mediated inhibitory effect on stellate cells, which become activated, leading to extracellular matrix deposition (Figure 1) (21). Another mechanism involved in this crosstalk is altered chemokine signaling in LSECs. Whereas after acute injury the endothelium secretes regenerative factors in a CXCR7-dependent manner, chronic injury shifts the response to a CXCR4-driven profibrogenic and pro-angiogenic program (22). Activated HSCs produce VEGF and angiopoietins, which is partly regulated by hedgehog signaling (23).

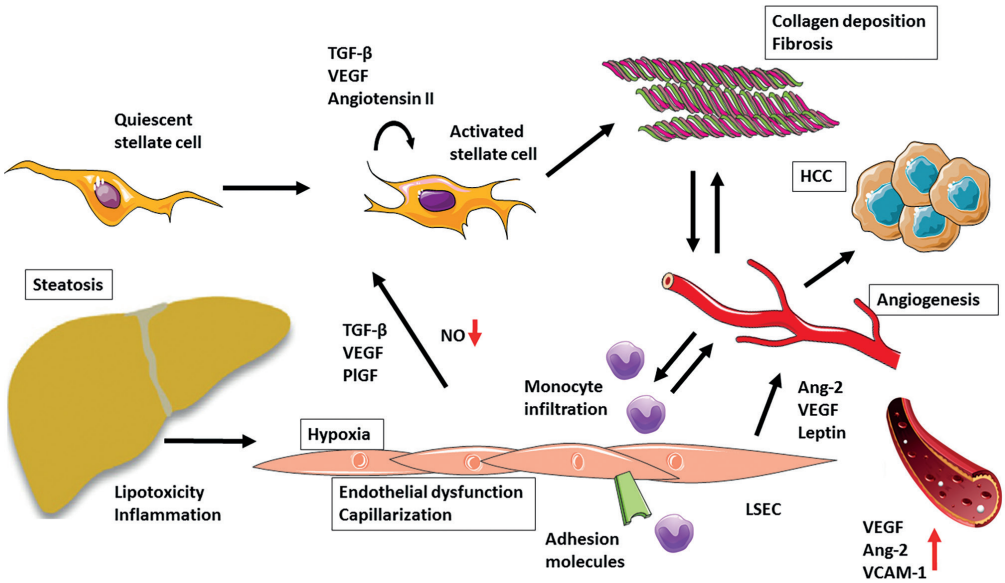

Figure 1. — Endothelial cells and angiogenesis in NASH-related fibrosis. Hepatic steatosis, lipotoxicity, hypoxia and inflammation cause phenotypical changes in sinusoidal endothelial cells (LSECs), leading to endothelial dysfunction and capillarization. Instead of NO, which maintains stellate cell (HSC) quiescence, LSECs release fibrogenic and angiogenic mediators, thereby activating HSC. Simultaneously, angiogenic factors released by LSECs, leukocytes and HSC induce hepatic angiogenesis. As shown by the effect of anti-angiogenic therapies, there is a close correlation between the progression of angiogenesis and fibrosis. Moreover, angiogenesis promotes the development and growth of HCC. Serum biomarkers such as VEGF, Ang-2 and VCAM-1 are elevated in patients with NASH and/or fibrosis. Ang-2, angiopoietin-2, HCC, hepatocellular carcinoma, NO, nitric oxide, PlGF, placental growth factor, VCAM-1, vascular cellular adhesion molecule-1, VEGF, vascular endothelial growth factor.

Leukocyte inflammation is another vital pathway in steatohepatitis. During NASH, monocytes infiltrate the liver and differentiate into monocyte-derived macrophages, which accumulate in fibrotic areas and stimulate angiogenesis through the production of VEGF and matrix metalloproteinase 9 (24,25). Using a single-cell sequencing approach, Ramachandran et al. recently confirmed that scar tissue-associated macrophages upregulate lipid metabolic, fibrogenic as well as pro-angiogenic pathways (26).

Markers of angiogenesis are elevated in human NAFLD

Pathological angiogenesis has been described in chronic liver disease, irrespective of etiology, and might be the evolutionary result of adaptive responses to acute liver injury. For instance, LSECs and pro-angiogenic factors dynamically orchestrate liver regeneration after partial hepatectomy. In a first phase, endothelial Ang-2 levels are downregulated, thereby promoting hepatocyte proliferation. During a second, angiogenic, growth phase, Ang-2 rapidly increases and stimulates the expression of VEGF receptor 2, enabling regenerative angiogenesis (27).

To unravel the pathophysiology and identify potential biomarkers, several studies have assessed the serum levels and/or hepatic expression of angiogenic factors in patients with NAFLD. Although circulating angiogenic factors in general were found to be elevated, reports on levels of individual factors have sometimes been discordant. For example, elevated VEGF serum levels have been reported in NAFLD (28) or selectively in patients with NASH (29), whereas this has not been confirmed in other cohorts (30,31). A macro-array gene expression analysis on the liver of obese patients with NASH showed that VEGF as well as inflammatory genes were overexpressed compared to obese controls without NASH (32), which was validated in a second cohort (28). Similar observations have been published on the endothelial dysfunction marker asymmetric dimethyl arginine (33). Finally, soluble forms of the adhesion molecules vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule-1 and vascular adhesion protein (VAP)-1, which mediate leukocyte infiltration, correlate with histological disease severity (34-36). Notably, VCAM-1 could serve as a biomarker of fibrosis severity in NAFLD (35), if validated, and is also a potential therapeutic target. Inhibition of the VCAM-1 ligand VLA-4 inhibited monocyte infiltration into the liver, improving both hepatic inflammation and glucose tolerance (37).

In patients with NAFLD, the development of steato-hepatitis is accompanied by an increased hepatic microvessel density and the emergence of centrizonal arteries (38-40). Despite the elevations in serum markers of angiogenesis in patients with NAFLD described above, we have recently confirmed earlier reports that new microvessels develop specifically in individuals with NASH, in proximity to foci of inflammation and hepatocellular ballooning (40). Furthermore, the extent of hepatic angiogenesis correlates strongly with the degree of liver fibrosis in NASH (38,39), which is similarly observed in other chronic liver diseases.

Importantly, population-based studies have reported that NAFLD increases the incidence of fatal and non-fatal cardiovascular events. A meta-analysis on more than 30000 patients confirmed that NAFLD was associated with a 64% increased risk for these events, and that patients with more severe NAFLD (defined in various ways, for example increased fibrosis stage on biopsy or high non-invasive fibrosis risk scores) had an even higher risk (41).

Considering the complex multisystem nature of metabolic disease, a myriad of pathophysiological mechanisms are implicated (42). Endothelial dysfunction, angiogenesis and vascular alterations are likely involved. For instance, the hepatic and systemic endothelial dysfunction that manifests itself, as discussed, in a reduced arterial vasodilation and elevated levels of circulating markers, can contribute to atherosclerosis. Whereas endothelial progenitor cells facilitate vascular repair after endothelial injury, their numbers are decreased in patients with NAFLD (43). Moreover, various papers found a link between NAFLD and an increase in prothrombotic factors. The strongest association was found for plasminogen activator inhibitor-1 (44), a key marker of endothelial dysfunction that promotes the formation of prothrombotic microparticles (45).

Angiogenesis drives inflammation and fibrosis in NAFLD

In accordance with clinical data, pathological angiogenesis has been observed in multiple animal models for NASH, including the methionine-choline deficient (MCD) diet, streptozotocin/western diet and db/db mouse models, and the choline-deficient, amino acid-defined rat model (40,46,47).

There is a close spatial association between angiogenesis and liver fibrosis. HSCs located in developing fibrotic septa express pro-angiogenic mediators (VEGF, Ang-1) in a hypoxia-dependent manner. In established bridging septa however, the expression of these mediators was shown to be limited to the edges, indicating a specific involvement in active fibrogenesis (48,49). NASH is characterized by perisinusoidal fibrosis, especially in zone 3. Interestingly, angiogenesis in NASH patients as well as in a rat model of NASH was likewise present in this region (40,46).

Functionally, angiogenesis promotes hepatic inflammation and fibrosis, as various inhibitors of angiogenesis reduce the latter. For instance, angiotensinogen II receptor blockers, which impact cardiovascular physiology and are used in the treatment of hypertension, decreased liver

fibrosis and angiogenesis in a rat NASH model (50,51). Leptin is an adipokine that induces satiety and thus regulates appetite. Leptin deficiency, as in ob/ob mice or Zucker rats, causes hyperphagia and obesity. The leptin receptor is also expressed in endothelial cells, and its activation induces both angiogenesis and an upregulation of TGF-β (52). Consequently, Zucker rats are protected from fibrosis, HCC and angiogenesis compared to control rats, despite a similar severity of underlying NASH (46). In general, this pathway could account for the resistance to fibrosis development in leptin-deficient animals (53). Our lab has previously shown that treatment with anti-VEGF receptor 2 antibodies could reduce experimental steatohepatitis, angiogenesis and the expression of fibrogenic mediators (47). More recently, we focused on the vascular destabilizing factor Ang-2, as patients with NASH had elevated Ang-2 serum levels compared to controls and patients with NAFL, which also correlated with the degree of hepatic angiogenesis. Inhibiting the interaction between Ang-2 and its receptor Tie2 with the peptibody L1-10 reduced hepatic inflammation and fibrosis in two NAFLD mouse models, and partially normalized the disorganized vascular network with sinusoidal obliteration and sprouting angiogenesis found in mice fed the MCD diet (Figure 2) (40). These changes were in large part mediated by LSECs, given that the expression of Ang-2 and Tie2 is mostly restricted to these cells (54), and that treatment reduced the expression of pro-inflammatory mediators in isolated endothelial cells as well as in an *in vitro* endothelial cell line (40).

An extensive cellular communication network underlies these observations. LSEC-derived TGF-β and angiogenic factors, especially placental growth factor (PlGF) and VEGF, stimulate HSC activation (49,55). Furthermore, TGF-β and angiotensinogen II upregulate VEGF in HSC themselves (50), leading to a loop in which autocrine and paracrine VEGF induces HSC migration, proliferation and matrix deposition (56) (Figure 1). VAP-1 is also expressed by both LSECs and HSCs and promotes HSC activation and migration. *In vivo*, VAP-1 deficiency or inhibition suppressed fibrosis progression in models of NASH and carbon tetrachloride-induced liver disease (36). Conversely, in one study, restoration of LSEC differentiation was able to reverse HSC activation and mild fibrosis (57).

An antifibrotic effect of anti-angiogenic molecules has also been reported in multiple models of other chronic liver diseases (55,58-61), further corroborating the crucial crosstalk between angiogenesis and fibrogenesis, mediated by HSC activation and LSEC dysfunction, in liver disease progression.

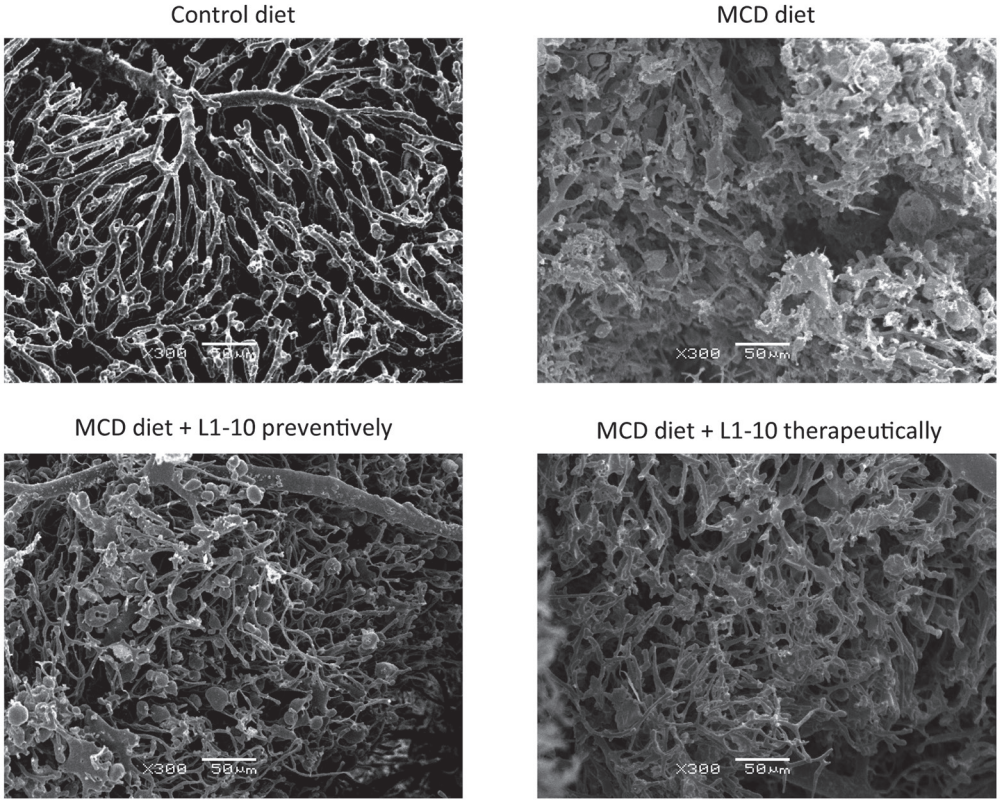

Figure 2. — Scanning electron microscopy of hepatic vascular corrosion casts. MCD diet feeding results in a severe distortion of the microvascular architecture, with sinusoidal obliteration and the formation of vascular blebs indicative of sprouting angiogenesis. These changes were partially prevented or reversed following treatment with the Ang-2 inhibitor L1-10. Original magnifications, 300x. Reproduced with permission from (40).

NASH-HCC is fueled by pathological angiogenesis

HCC is a major complication of chronic liver disease. In the setting of NASH, HCC can develop in the absence of cirrhosis, although advanced fibrosis is most often present. NASH is a rising cause of HCC, and the incidence of NASH-induced HCC in the US is estimated to more than double between 2015 and 2030 (4).

HCCs grow rapidly and are therefore consistently in need for oxygen and nutrients. Thus, HCCs induce angiogenesis and are generally hypervascular (12). Notably, fast-growing HCCs display a distinctive gene expression profile, characterized by the overexpression of genes involved in angiogenesis and endothelial cell migration (62). In this study, Ang-2 was the most significantly upregulated gene in a signature able to predict HCC growth and survival. Other studies have also confirmed elevated Ang-2 levels in patients with HCC (63,64). Moreover, intratumoral Ang-2 expression is higher than in the surrounding tissue (65). Experimental evidence suggests that Ang-2 might also be a therapeutic target in HCC. The use of Ang-2 overexpressing HCC cells in a xenograft model increased the vessel density and tumor growth compared to mice injected with untransfected or Ang-1 overexpressing cells (66). In accordance, we have shown that Ang-2 inhibition countered the development of HCC on a background of NASH (40). Other anti-angiogenic compounds have similarly shown benefit in mouse models of NASH-HCC. For instance, the angiotensin II receptor blocker telmisartan prevented HCC formation in the amino acid-defined rat model (51). It is still unclear to what extent these anti-angiogenic drugs have a direct antitumor effect, as a dampening of the underlying fibrosis and inflammation may also contribute to reduced HCC progression.

These findings are in line with clinical evidence in HCC (not specific for NASH-HCC). Most pharmacological agents currently approved for the treatment of advanced HCC work at least in part as angiogenesis inhibitors. Sorafenib, regorafenib and lenvatinib are tyrosine kinase inhibitors that inhibit a broad array of receptors, including the VEGF receptors 1-3, and for regorafenib, the Ang-2 receptor Tie2. These compounds have demonstrated modest survival benefits in phase III trials for 1st (sorafenib, lenvatinib) or 2nd line (regorafenib) (67-69). Ramucirumab is a monoclonal antibody specific for the VEGF receptor 2 and has shown a survival benefit in 2nd line in patients with α-fetoprotein levels >400 ng/mL (70). For a recent review on this topic, we refer to Morse et al. (71)

Conclusion

Early changes at the stage of NAFL include endothelial dysfunction and tissue hypoxia. As NAFLD progresses to NASH, these factors aggravate, and together with leukocyte inflammation and stellate cell activation, induce vascular expansion. Angiogenesis is tightly coupled to the progression of liver fibrosis and HCC, both in NAFLD as well as other chronic liver diseases. Although proof-of-concept studies are promising, the challenge of translation to specific strategies for the diagnosis and/or treatment of NASH and liver fibrosis remains.

Disclosures

The authors report no disclosures relevant to this work.

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Figure 1 was in part adapted from material made available by Smart – Servier Medical Art (https://smart.servier.com/).

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