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Review

Angiogenesis in liver disease☆

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Angiogenesis and disruption of liver vascular architecture have been linked to progression to cirrhosis and liver cancer (HCC) in chronic liver diseases, which contributes both to increased hepatic vascular resistance and portal hypertension and to decreased hepatocyte perfusion. On the other hand, recent evidence shows that angiogenesis modulates the formation of portal-systemic collaterals and the increased splanchnic blood flow which are involved in the life threatening complications of cirrhosis. Finally, angiogenesis plays a key role in the growth of tumours, suggesting that interference with angiogenesis may prevent or delay the development of HCC. This review summarizes current knowledge on the molecular mechanisms of liver angiogenesis and on the consequences of angiogenesis in chronic liver disease. On the other hand, it presents the different strategies that have been used in experimental models to counteract excessive angiogenesis and its potential role in preventing transition to cirrhosis, development of portal hypertension and its consequences, and its application in the treatment of hepatocellular carcinoma.

***Keywords*: Angiogenesis; Liver fibrosis; Portal hypertension; Liver cancer; Liver regeneration**

1. Introduction

Cirrhosis and hepatocellular carcinoma (HCC) are common lethal diseases in European countries, together representing the third cause of death in adults over 50 years old, as well as the indication for over 90% of the 5.000 liver transplants that are performed every year within the EU. These features are increasing due to the consequences of the hepatitis C epidemic in the 70's. Thus, its socioeconomic impact is extraordinary.

The formation of new vessels (angiogenesis) and the establishment of an abnormal angioarchitecture of the liver is a process strictly related to the progressive fibrogenesis leading to cirrhosis and liver cancer. Investigation into these aspects is complex and certainly requires a joint effort of a multidisciplinary team of basic investigators, pathologists, and hepatologists in the areas of liver fibrosis, hepatic circulation, and portal hypertension and its complications.

Established evidence clearly indicates that chronic liver diseases are characterized by intrahepatic vascular remodelling with capillarization of sinusoids, fibrogene-

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Abbreviations: CLDs, chronic liver diseases; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; ECM, extra-cellular matrix; VEGF, vascular endothelial growth factor; HSC, hepatic stellate cells; MFs, myofibroblasts; PVL, portal vein ligation; LSECs, liver sinusoidal endothelial cells; HGF, hepatocyte growth factor; HCC, hepatocellular carcinoma.

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sis and development of intrahepatic shunts, which would lead to increased hepatic resistance (and hence to increased portal pressure) and decreased effective hepatocyte perfusion (and hence to liver failure). In addition, new original data obtained by the authors of this review suggest that vascular endothelial growth factor (VEGF)/ platelet-derived growth factor (PDGF) driven angiogenesis is of paramount importance in the formation of portal-systemic collaterals and of the hyperdynamic circulation which are responsible for the main complications of cirrhosis often leading to death: gastroesophageal varices, massive upper gastrointestinal bleeding, ascites, spontaneous bacterial peritonitis and hepatic encephalopathy. Finally, angiogenesis is known to play a critical role in the growth of tumours, which makes it plausible to hypothesize that early interference with angiogenesis signalling may prevent the transition from hepatic dysplasia to HCC.

This article reviews the translational research effort that has been made recently on both the molecular mechanisms and signal transduction cascade of liver angiogenesis, and the consequences of angiogenesis in chronic liver disease, emphasizing studies exploring different strategies to counteract excessive angiogenesis to prevent progression of liver fibrosis and transition to cirrhosis in chronic hepatitis, to prevent the development of portal hypertension and its consequences, and finally to prevent the formation and growth of hepatocellular carcinoma often occurring in patients with cirrhosis.

2. Angiogenesis and fibrogenesis

Pathological angiogenesis, irrespective of the aetiology, has been indeed extensively described in chronic liver diseases (CLDs) characterized by an extensive and prolonged necro-inflammatory and fibrogenic process, including chronic HBV, HCV and autoimmune hepatitis [1,2], and primary biliary cirrhosis [3]. The formation of new vessels, which is closely associated with the pattern of fibrosis development typical of the different CLDs [4], leads to the progressive formation of the abnormal angio-architecture distinctive of cirrhosis, i.e. the common end-point of fibrogenic CLDs. Accordingly, the association of fibrogenesis and angiogenesis should be regarded as crucial in the modern evaluation of disease progression and in the search for therapeutic targets. In addition, depending on the different pattern of fibrogenic evolution (i.e. post-necrotic, biliary, centrolobular, pericellular/perisinusoidal), the extent of neo-angiogenesis may have profound consequences on the rate of disease progression to cirrhosis and represents a key determinant affecting reversibility of fibrosis (Table 1).

From a mechanistic point of view, angiogenesis in fibrogenic CLDs can be interpreted according to two main pathways. First, the process of liver chronic wound healing typical of fibrogenic CLDs is characterized by an over-expression of several growth factors, cytokines and metalloproteinases (MMPs) with an inherent pro-angiogenic action [5]. In particular, platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) have been shown to exert a potent pro-fibrogenic and pro-angiogenic role. In addition, an increased gene expression of integrins, β-catenin, ephrins, and other adhesion molecules involved in extra-cellular matrix (ECM) remodelling and angiogenesis has been clearly demonstrated in CLDs [6,7]. Second, neo-angiogenesis is stimulated in hepatic tissue by the progressive increase of tissue hypoxia. This mechanism is strictly linked to the anatomical modifications following the establishment of periportal fibrosis with an increased contribution of the hepatic artery to the formation of sinusoidal blood [8]. Accordingly, sinusoidal blood flow becomes increasingly arterialized with hepatocytes adjusting to an abnormally high oxygen concentration. Subsequently, the progressive capillarization of sinusoids leads to an impairment of oxygen diffusion from the sinusoids to hepatocytes with the consequent up-regulation of pro-angiogenic pathways [9–11].

Although neo-angiogenesis is a common feature of most chronic inflammatory and fibrogenic disorders [12,13], hepatic angiogenesis may substantially differ from homologous processes in other organs or tissue on the basis of: (a) the rather unique phenotypic profile and functional role of activated hepatic stellate cells (HSC) and of other liver myofibroblasts (MFs) [14–20] (b) the presence of two different microvascular structures described (i.e., sinusoids lined by fenestrated endothelium versus large vessels lined by a continuous one); (c) the existence of ANGPTL3, a liver specific angiogenic factor [21]. Evidence obtained from morphological studies suggests that angiogenesis occurring in hepatic tissue undergoing chronic wound healing is characterized by branching of neo-vessels from the existing vasculature. The large majority of these neo-vessels originate from the fine portal vein branches and tend to establish a connection between the portal system and the hepatic veins [8,22]. The role of bone marrow-derived endothe-

Table 1
Reversibility of liver fibrosis according to the pattern.

Fibrosis pattern	Early portal to central septa	Neo angiogenesis	Reversibility
Post-necrotic	++++	++++	+
Biliary	+	++	++++
Centrolobular	–	±	++++
Pericellular-perisinusoidal	–	++	+++

lial precursors (vasculogenesis) in hepatic angiogenesis has been suggested by studies employing animal models of hepatic fibrogenesis [23,24] and needs to be substantiated in human CLDs.

A key area in the study of the cellular and molecular relationships existing between fibrogenesis and angiogenesis concerns the pro-angiogenic role of activated HSC and other ECM-producing cells such as portal fibroblasts and myofibroblasts. Hypoxic conditions, through the involvement of the transcription factor HIF-1α, are able to up-regulate expression of VEGF [2,17,25–27] and angiopoietin I [17,26] in rat or human HSC. Moreover, exposure to hypoxia results in up-regulation of VEGF receptors type I (Flt-1) and type II (Flk-1) as well as of Tie-2 (i.e., the receptor for angiopoietin I) in the same cell type [11,17,25]. Hypoxia-dependent up-regulation and release of VEGF by human HSC/MFs can stimulate, in a paracrine and/or autocrine manner, non-oriented migration and chemotaxis of human HSC/MFs [17]. This feature depends mainly on the interaction between VEGF and Flk-1 and may explain the significant "*in vivo*" anti-fibrotic effect reported in an experimental model in which animals were treated with neutralizing anti-Flk-1 antibodies [28]. Recent "*in vivo*" data obtained in human and rat fibrotic/cirrhotic livers, indicate that α-SMA-positive cells (i.e., myofibroblast-like phenotype) expressing VEGF, Ang-1 or the related receptors Flk-1 and Tie-2, are consistently localized at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa [17]. This distribution may reflect two different phases of angiogenic process during chronic wound healing: an early phase, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by ECM-producing cells, and a later phase occurring in larger and more mature fibrotic septa where the chronic wound healing is less active and fibrogenic transformation more established. In this latter setting pro-angiogenic factors are expressed only by endothelial cells, a scenario that is likely to favour the stabilization of the newly formed vessels. In this context, it is relevant that the promotion of a pro-angiogenic phenotype in activated HSC is stimulated also by non-hypoxic conditions and particularly by the exposure to the pro-fibrogenic adipokine leptin [26].

An elegant and convincing demonstration of the interplay between inflammatory response, angiogenesis, and fibrogenesis has been recently provided by an experimental study in which all these features have been significantly reduced by the treatment with the multitargeted receptor tyrosine kinase inhibitor sunitinib [29]. This study is of relevance because it provides evidence for a possible dual and converging pharmacological action (i.e. anti-fibrogenic and anti-angiogenic) able to interfere directly with liver myofibroblasts, presumably by negatively affecting PDGF-dependent signalling.

3. Role of angiogenesis in portal hypertension

Portal hypertension is a major complication of cirrhosis of the liver, which represents a leading cause of death and liver transplantation [30–32]. A salient feature of portal hypertension is the formation of an extensive network of portosystemic collateral vessels, which include the oesophageal and gastric varices, responsible for variceal bleeding, associated with a high mortality rate [30–32]. In addition, collateral vessels result in shunting of portal blood into the systemic circulation, causing high systemic concentrations of several substances normally metabolized by the liver, such as drugs, toxins, hormones, and bacteria. These in turn contribute to severe complications of cirrhosis, including portosystemic encephalopathy and sepsis [30–32]. Therefore, successful design of medical treatment for portal hypertension requires a better understanding of the mechanisms underlying the formation of portosystemic collateral vessels, an issue that has remained largely unexplored. Traditionally, formation of collaterals was considered to be a mechanical consequence of the increased portal pressure that will result in the opening of these vascular channels. Accordingly, therapeutic strategies are mainly aimed at decreasing portal pressure [30–32]. However, as discussed in this article, recent studies have examined another approach, based on the potential involvement of angiogenesis in the development of these collateral vessels.

Another characteristic feature of the portal hypertensive syndrome is the development of a hyperdynamic circulatory state, with an increase in blood flow in splanchnic organs draining into the portal vein and a subsequent increase in portal venous inflow [30–32]. Such an increased portal venous inflow represents a significant factor maintaining and worsening portal hypertension [30–32]. The mechanisms underlying this splanchnic hyperemia are not fully understood, but involve overproduction of endogenous vasodilators and decreased vascular reactivity to vasoconstrictors [30–32]. An intriguing possibility is that an increased formation of splanchnic blood vessels through active angiogenesis could also be involved in the maintenance of a hyperdynamic splanchnic circulation in portal hypertension.

In the last few years, these possibilities have been addressed by studying the effects of different anti-angiogenic strategies aimed at inhibiting the signalling pathways of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and placental growth factor (PLGF), which are essential mediators

of angiogenesis [33–35], on the development and maintenance of hyperdynamic splanchnic circulation and portosystemic collateralization in experimental models of portal hypertension.

3.1. Increased angiogenesis in portal hypertension

Evidence supporting a role for angiogenesis in the pathogenesis of portal hypertension includes recent investigations demonstrating that VEGF, a potent angiogenic factor [35], is overexpressed in splanchnic organs from portal hypertensive animals (Fig. 1) [36,37]. The expression of VEGF receptor-2 (VEGFR-2) and the endothelial cell marker CD31 [38] is also increased in the splanchnic territory in experimental models of portal hypertension [36,37]. These and other studies provided evidence of increased VEGF-driven splanchnic angiogenesis in portal hypertensive animals and in cirrhotic patients [39–42].

The precise mechanism triggering VEGF-dependent angiogenesis in portal hypertension remains speculative, but it is likely to be multifactorial. Indeed, several factors relevant to the pathogenesis of portal hypertension, such as tissue hypoxia, cytokines, and mechanical stress, have been shown to promote VEGF expression in various cell types and tissues [30–32,35].

3.2. VEGF signalling blockade in portal hypertension

Recent studies have determined the effects of several angiogenesis inhibitors, with different modes of action, in experimental models of portal hypertension. These studies demonstrated that treatment with an anti-VEGFR-2 monoclonal antibody (DC101) [43] from the induction of portal hypertension markedly decreased the formation of portosystemic collateral vessels and reduced splanchnic vascularization in portal hypertensive mice [36]. Similar results were obtained using SU5416, a specific inhibitor of the tyrosine kinase domain of VEGFR-2 [37,44] as an anti-angiogenic strategy, which caused a significant 52% decrease in the extent of portosystemic collateral formation in rats with partial portal vein ligation (PPVL) [37]. In addition, SU5416 also markedly reduced the splanchnic hyperdynamic circulation in these animals, indicating that increased splanchnic arteriolar bed size mediated by a VEGF-dependent angiogenic process significantly contributes to the increased portal blood inflow. Portal pressure however was not modified, most likely because of the concomitant inhibition in the formation of portosystemic collateral vessels [37]. These findings were further confirmed using rapamycin, which inhibits VEGF production and reduces portosystemic collateral vessel formation by 67% in portal hypertensive rats, in parallel with a significant attenuation of the hyperdynamic splanchnic circulation [45].

Taken together, these data indicate that the development of hyperdynamic splanchnic circulation and splanchnic neovasculature as well as the formation of portosystemic collateral vessels in portal hypertensive animals are in part VEGF-dependent angiogenic processes that can be significantly prevented by inhibitors of the VEGF/VEGF receptor-2 signalling pathway, when its administration was started at the time of portal hypertension initiation [36,37,45].

3.3. Combined VEGF and PDGF signalling blockade

The studies described so far highlight the importance of angiogenesis in the pathogenesis of portal hypertension and suggest that anti-angiogenic treatment might be a promising therapeutic strategy to prevent the progression of the portal hypertensive syndrome [36,37,45]. In clinical practice, however, portal hypertension does not represent a significant problem until it is quite advanced and associated with clinical manifestations. Thus, for anti-angiogenic treatment strategies to become of great clinical relevance these should be able to revert the circulatory abnormalities associated with portal hypertension once these are fully developed [45].

In this regard, it should be noted that in the process of neovascularization, VEGF plays a predominant role in the initial stages of formation of new blood vessels, activating the proliferation of endothelial cells and the subsequent formation of an endothelial tubule, while maturation of the newly formed vessels is mainly modulated by the proangiogenic growth factor platelet-derived growth factor (PDGF), which regulates the investiture of the endothelial tubule with mural cell and pericyte populations, thereby stabilizing the vascular architecture of the nascent vessel [33,46]. Based on these considerations, it was hypothesized that the simultaneous targeting of the VEGF and PDGF signalling pathways, that is the simultaneous targeting of endothelial cells and pericytes, could provide a greater vascular destabilization and a better vascular regression than targeting either alone.

It was first demonstrated that development of portal hypertension in PPVL rats was associated with a progressive overexpression of PDGF, which reached its peak later in the course of portal hypertension than VEGF overexpression [45]. In accordance with the working hypothesis, the continued administration of the VEGF signalling inhibitor rapamycin plus the PDGF signalling inhibitor Gleevec® markedly decreased the splanchnic neovascularization and the pericyte coverage of neovessels in portal hypertensive rats (Fig. 2) [45]. This combined treatment also resulted in a virtually complete reversal of the increased portal pressure (40% reduction) and the increased portal venous blood inflow of these animals (Fig. 3). This is important since clinical studies have shown a dramatic

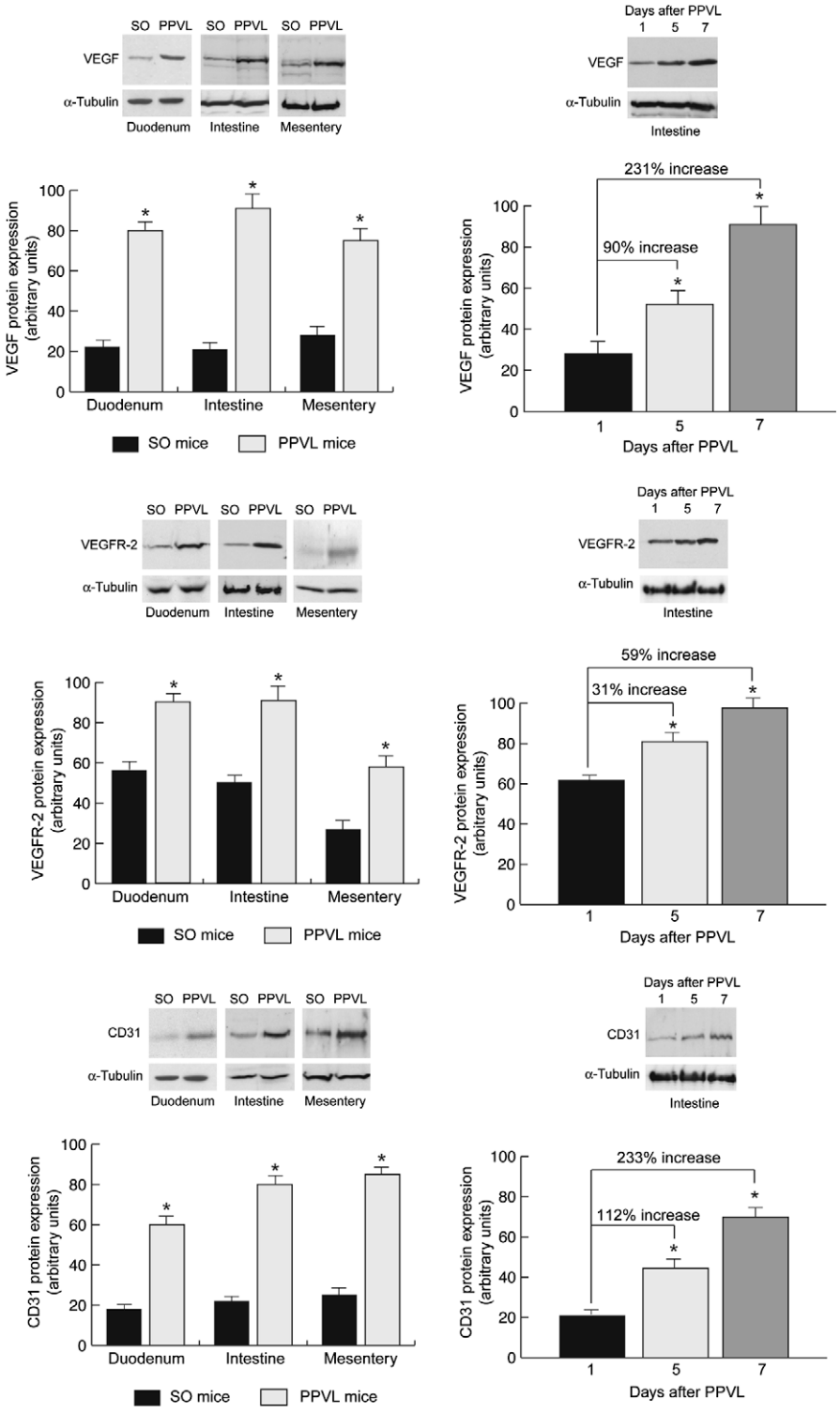

Fig. 1. Overexpression of VEGF, VEGF receptor-2, and CD31 in portal hypertensive mice. (Left) Protein expressions in splanchnic organs from partial portal vein-ligated (PPVL) mice and sham-operated (SO) control animals, seven days after the initial surgery. (Right) Protein expressions at days 1, 5, and 7 after the induction of portal hypertension in mice. Representative western blots are shown at the top and densitometric quantification normalized to α-tubulin is shown at the bottom of each panel. $^{*}P < 0.05$ vs. SO mice (left) or vs. day 1 (right).

reduction of the risk of portal hypertensive complications and improved survival in patients achieving a decrease in portal pressure of at least 20% under drug therapy. Notably, the magnitude of the effects of the combination treatment was superior than the addition of the effects of either drug alone, suggesting a synergis-

tic regulatory interaction between the VEGF and PDGF signalling pathways in mediating the maintenance of the vascular and hemodynamic abnormalities observed in portal hypertensive rats [45]. These findings further suggest that in the absence of proliferating perivascular cells (ie, after PDGF signalling inhibition), the endothelium is more vulnerable to anti-angiogenic therapies targeting endothelial cells, such as VEGF signalling blockade [47].

Overall, these findings demonstrate that angiogenesis is a pathological hallmark of portal hypertension, and have made the control of new blood vessel formation a promising therapeutic target to prevent the progression and to promote the regression of portal hypertension-related complications in clinical scenarios.

3.4. Role of PLGF-derived angiogenesis in portal hypertension

Placental growth factor (PLGF) was originally discovered in human placenta in 1991 and is a member of the VEGF family. Alternative splicing of the human PLGF gene generates three isoforms (PLGF-1, PLGF-2, and PLGF-3), while in mice only PLGF-2 is present [48]. The 3-dimensional structures of VEGF and PLGF are similar; however they have only 42% identical amino-acids [48,49].

PLGF is not highly expressed in normal tissue and during embryogenesis, as PLGF-deficient mice are viable and fertile and do not display major abnormalities [49–53]. In the healthy state, PLGF plays a role during pregnancy and during the ovarian cycle, without affecting quiescent vessels. However, loss of PLGF impairs angiogenesis in the wounded skin, ischemic retina, limb, heart and in cancer, whereas administration of recombinant PLGF (rPLGF) promotes collateral vessel growth in models of limb and myocardial ischemia [50,54].

Placental growth factor binds only to VEGFR-1 and neuropilin-1 and enhances the effects of VEGF and thus angiogenesis only under pathological conditions [54–56]. VEGFR1 is minimally expressed in adult quiescent ves-

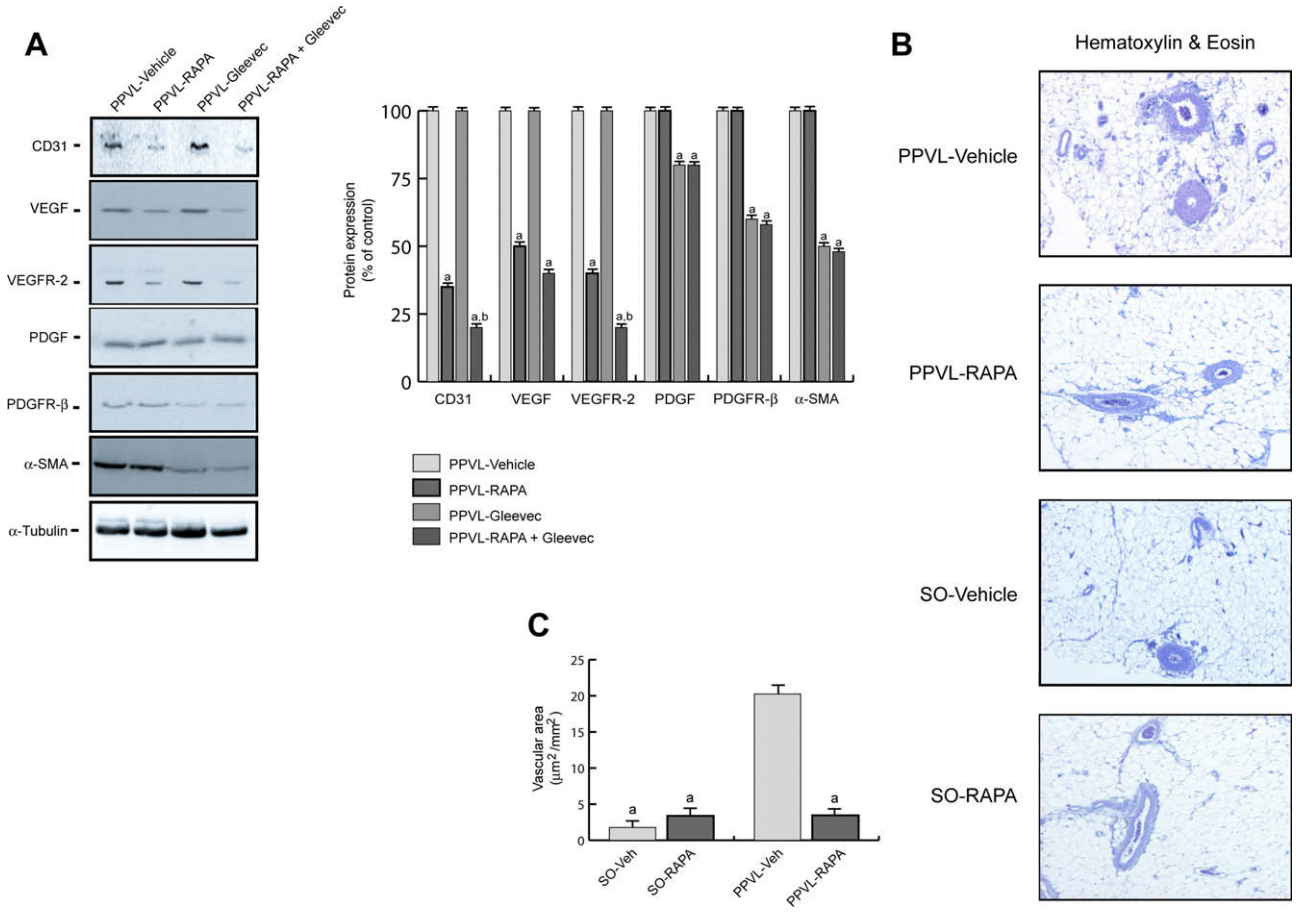

Fig. 2. Effects of rapamycin (RAPA), Gleevec or RAPA + Gleevec on angiogenesis mediators when portal hypertension was completely established. (A) Expression of CD31, VEGF, VEGFR-2, PDGF, PDGFR-beta, and α-smooth muscle actin (α-SMA) in the intestine of PPVL rats after treatment with RAPA, Gleevec, RAPA + Gleevec or vehicle. Representative blots are shown at the left and quantification of expression normalized to α-tubulin is shown at the right. (a) $P < 0.05$ vs. PPVL-vehicle. (b) $P < 0.05$ vs. PPVL-RAPA. (B) Representative histological images of mesentery sections stained with H&E from PPVL and SO rats treated with RAPA or vehicle. Original magnification 40×. (C) Quantitative analysis of neovascularization in the mesentery from PPVL and SO rats treated with RAPA or vehicle. (a) $P < 0.05$ vs. PPVL-vehicle.

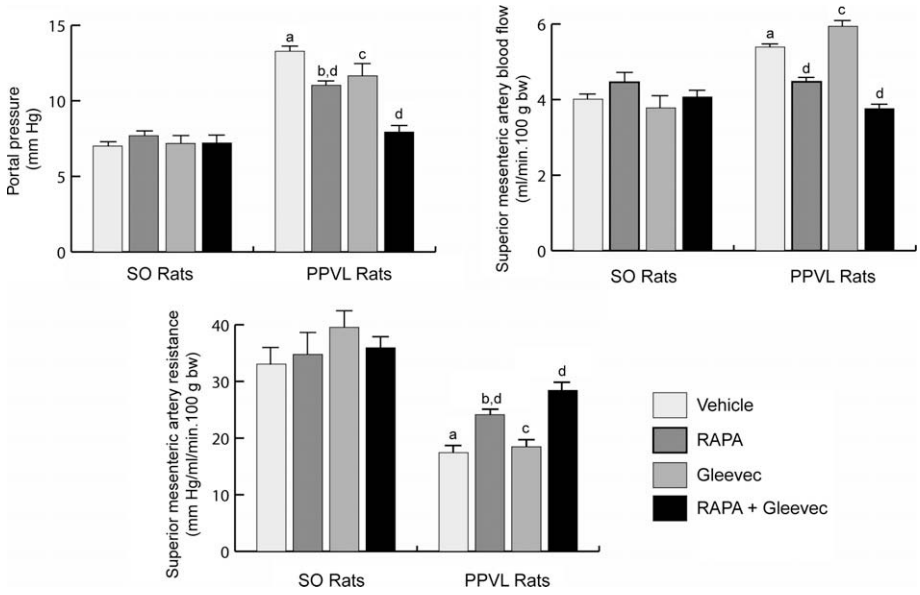

Fig. 3. Effects of RAPA, Gleevec or RAPA + Gleevec on splanchnic hemodynamics when portal hypertension was completely established. (a) $P < 0.05$ vs. SO-vehicle; (b) $P < 0.05$ vs. SO-RAPA; (c) $P < 0.05$ vs. SO-Gleevec; (d) $P < 0.05$ vs. PPVL-vehicle.

sels but membranous localisation is markedly up-regulated during pathological conditions, stimulating the PLGF-dependent angiogenic signals [55]. This makes PLGF attractive as a therapeutic target [49,55]. The synergistic effects between PLGF and VEGF are shown in Fig. 4.

3.5. Effects of the PLGF/VEGFR1 signalling pathway

PLGF reconstitutes haematopoiesis by recruiting VEGFR1 positive stem cells from the bone marrow, stimulates the survival of endothelial cells and monocytes [56–58], and plays an important role in inflammation by recruiting endothelial cells, monocytes, progenitor cells and functional natural killer cells and by stimulating migration of inflammatory cells and chemotaxis. Leukocytes and inflammation can cause the release of multiple angiogenic factors such as VEGF, platelet-derived growth factor (PDGF), PLGF and basic fibroblast growth factor [59].

PLGF is also a major player in arteriogenesis (and mature vessel formation) via recruitment of bone marrow cells, smooth muscle cells, pericytes, endothelial cells and monocytes [60]. PLGF enhances collateral growth by stimulating endothelial and smooth muscle cell growth. Other angiogenic factors such as VEGF, PDGF, and angiopoetin 1 enhance the formation of collaterals [51,59–63].

3.6. PLGF in portal hypertension

Geerts et al. have recently demonstrated that neoangiogenesis in the mesentery of portal hypertensive

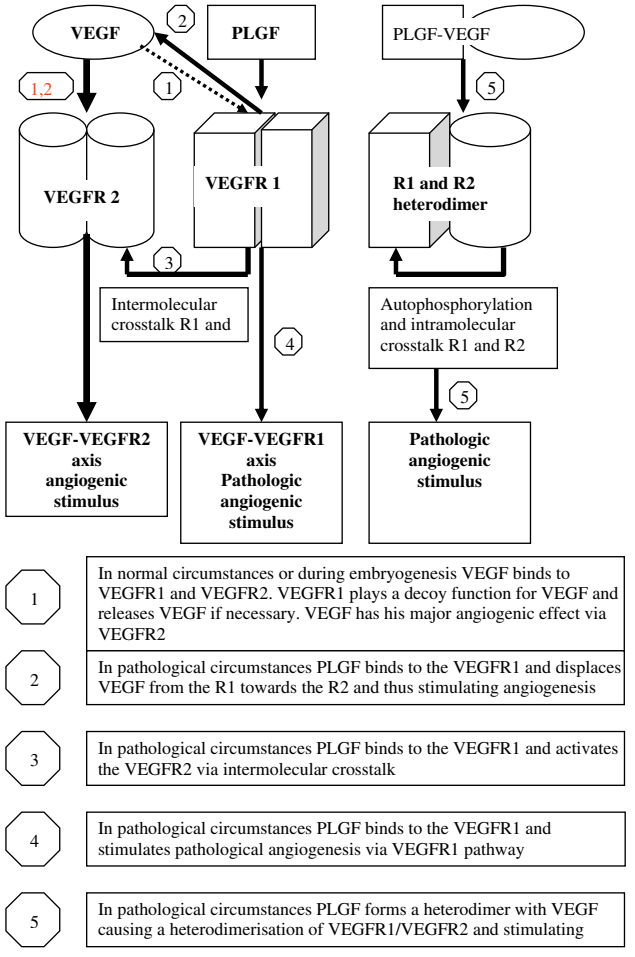

Fig. 4. Diagramm illustrating the synergism between vascular endothelial growth factor (VEGF) and placental growth factor (PLGF). (VEGF receptor 1, VEGFR1; VEGF receptor 2, VEGFR2).

mice is associated with an up-regulation of VEGF, PLGF protein levels, and CD31 (marker for endothelial cells and thus angiogenesis) [64,65].

Recent studies using PLGF knockout mice (PLGF−/−) have shown that PLGF(−/−) portal hypertensive mice do not develop mesenteric neo-angiogenesis. CD31 protein expression in portal hypertensive PLGF(−/−) mice was significantly lower than in portal hypertensive wild-type mice, and similar to those of sham operated mice [64,65]. This was also confirmed by immunohistochemistry. Portal hypertensive PLGF(−/−) mice showed a tendency towards a lower portal pressure and a significant decrease (but not normalization) in spleen weight [64,65]. These findings confirm that portal hypertension induces neo-angiogenesis and that this plays a role in the maintenance of the splanchnic hyperdynamic state. The lack of reduction in portal pressure in PLGF(−/−) mice is in accordance with previous observations inhibiting VEGF [36,37,45]. Further studies with anti-PLGF antibodies in the prevention and treatment of portal hypertension are underway. As the role of PLGF is especially restricted to pathological conditions it is a good target for therapy with potentially less severe side effects than the blockade of VEGF/PDGF.

4. Angiogenesis in liver regeneration

Much of our knowledge about vascular biology of the liver has come from studying angiogenesis during regeneration after partial hepatectomy. The different phases of physiological angiogenesis and sinusoidal remodelling during liver regeneration have been analyzed extensively. Dysregulation of the same pathways and processes in chronic liver disease leads to endothelial dysfunction, pathological angiogenesis, with formation of collaterals and eventually to portal hypertension. This section summarizes the timely, limited, highly orchestrated cellular and molecular interactions related to physiological angiogenesis during liver regeneration.

4.1. Role of liver sinusoidal endothelial cells in liver regeneration

The liver receives 25% of the total cardiac output, which arrives via the hepatic artery (1/3 of hepatic blood flow) and the portal vein (2/3 of hepatic blood flow). Blood flows through liver sinusoids, a unique microvasculature which consists of plates of liver sinusoidal endothelial cells (LSECs) between plates of hepatocytes, before coming in contact with the liver parenchyma. LSECs account for 20% of total liver cells (an estimated 1×10^8 cells) whereas hepatocytes represent the majority of liver cells (estimated 60% or 3×10^8 cells). LSECs have a unique phenotype in comparison to other organs characterized by a discontinuous, fenestrated endothelium, which lacks an organized basement membrane. Cellular cross-talk between LSECs and hepatocytes plays an important role in sinusoidal homeostasis and physiologic angiogenesis during liver regeneration and hepatic organogenesis.

Following partial hepatectomy (up to 70% removal), the liver will completely regenerate and reach its original functional mass with normal microscopic architecture, which is a unique capacity compared to all other organs with none or only limited regeneration. Experimental partial hepatectomy (PHx) involves the removal of two-thirds of the liver [66]. Within 8–10 days, the liver remnant enlarges, until the previous functional liver mass is restored. LSECs and hepatocytes in the normal liver are quiescent, with only 0.01–0.001% undergoing mitosis at any given time. PHx activates numerous transcription factors, induces the expression of more than 70 genes, and promotes cell cycle entry with synchronous proliferation of almost all hepatocytes by 24–36 h post-PHx. The initial wave of hepatocyte proliferation is followed by a second wave of replication of non-parenchymal liver cells, including LSECs and HSC, which start to proliferate 48–72 h after resection peaking at day 4–5 [67].

Hemodynamically seen, PHx leads to an increased sinusoidal blood flow since the portal influx to the liver remnant remains unchanged [68]. Hemodynamic changes and shear stress might induce hepatocyte proliferation through activation of urokinase plasminogen activator, matrix remodelling, and subsequent release of preformed hepatocyte growth factor (HGF) [69]. The initial proliferation of hepatocytes leads to the formation of avascular clusters of hepatocytes, where the central cells reside outside the oxygen diffusion distance of 200 μm of a capillary [67,70,71]. This is accompanied by ultrastructural changes of the sinusoids, with external compression of the sinusoids by proliferating hepatocytes, decrease of sinusoidal fenestrations and endothelial porosity 72 h post-PHx [71]. In a next stage, hypoxia and hepatocyte signalling induce LSEC activation, proliferation, and migration of neighbouring LSECs into the avascular clusters [72,73]. This leads to separation of hepatocytes with subsequent recanalization and formation of patent sinusoids. Around day 6 after PHx (when the regenerating liver is approaching its preoperative functional mass), a wave of apoptosis in LSEC can be detected with a maximum at day 8 [70,74,75]. This is in contrast to hepatocytes which do not show increased apoptotic rates during the regenerative process [70]. Eight to 10 days following PHx, liver mass is fully reconstituted, and the sinusoidal architecture restored to normal.

Circulating endothelial progenitor cells (characterized by stem cell markers such as CD117 or CD133) have also been shown to play a role in angiogenesis during liver regeneration. Circulating endothelial cells are

mobilized from bone marrow by systemically circulating growth factors and chemokines produced in the regenerating liver and participate in this process by homing to sites of neovascularization in the liver and by committing to LSECs [76,77]. Treatment with exogenous VEGF increased mobilization of circulating endothelial progenitor cells and accelerated liver regeneration in mice after PHx [76].

4.2. Sinusoidal remodelling in the regenerating liver

Recent evidence suggests that an alternative mode of angiogenesis, called *intussusceptive angiogenesis* contributes to the angiogenic process in addition to the classical form of *sprouting angiogenesis* described above. Sprouting (angiogenesis from a pre-existing vascular bed) is dependent on endothelial cell proliferation with subsequent lumen formation in the sprout. In contrast to sprouting, angiogenesis by intussusception consists of microvascular remodelling by transcapillary pillar formation and relies much less on endothelial cell proliferation [78,79]. Growth of these endothelial pillars leads to sinusoidal multiplication by successive fusion and partitioning of the existing vascular lumen. Intussusceptive angiogenesis has been found to occur as early as 12 h after PHx in mice [80]. The split sinusoids increase in girth and undergo augmentation by day 3 to 4, concomitant with endothelial cell proliferation [80].

The role of HSC in liver regeneration and sinusoidal remodelling is less well studied. HSC are closely associated with LSEC and function as liver-specific pericytes (vascular mural cells of the hepatic sinusoids) [16,81]. They regulate vessel stabilization, maturation and sinusoidal remodelling by direct contact and paracrine interaction with LSECs such as secretion of VEGF [16]. Mediated by platelet-derived growth factor (PDGF) produced by hepatocytes and LSECs, resting HSC start to proliferate (peaking at 48–72 h) and become activated within the first 72 h after PHx [69,82–84]. In addition, HSC are thought to control remodelling of the extracellular matrix in the space of Disse during regeneration (i.e. deposition of collagen IV, fibronectin and laminin) which will influence function of LSECs [69,71,82]. Similar to circulating endothelial progenitor cells, bone marrow-derived cells have also been shown to commit to HSC during liver regeneration [85].

4.3. Hypoxia and vascular factors regulate angiogenesis during liver regeneration

VEGF is the most important growth and survival factor for endothelium. VEGF promotes proliferation of endothelial cells, acts as an anti-apoptotic factor and regulates vascular permeability by inducing fenestration in LSECs [86,87]. Furthermore, VEGF induces the expression of proteases like collagenase [88], matrix metalloproteinases [89], urokinase- and tissue-type plasminogen activators [90], which enable endothelial cells to breakdown the surrounding extracellular matrix in order to migrate and form new blood vessels. VEGF is constitutively expressed in hepatocytes at low levels [91]. VEGF production by hepatocytes and HSC rapidly increases during liver regeneration. of VEGF is induced by cytokines (i.e. IL-6) and hypoxia in the centre of the regenerative and avascular hepatocyte clusters [67]. Hypoxia activates the transcription factor hypoxia-inducible factor (HIF)-1, which in turn induces the expression of downstream target genes including cMet, EPO (erythropoietin), VEGF and VEGFR-1 (VEGF receptor 1) [92–94]. Hepatocyte production of VEGF peaks 48–72 h after PHx and is detected mainly in periportal hepatocytes [72,91]. Administration of VEGF in hepatectomized rodents increases LSEC and hepatocyte proliferation [91,95], accelerates gain in liver mass [96] and improves functional hepatic recovery [93]. Transduction of VEGF before hepatic resection also hastens functional hepatic recovery in mice with fatty liver, which is known for its impaired regenerative capacity [93]. Neutralizing antibodies against VEGF inhibit hepatocyte and endothelial cell proliferation after PHx [91].

VEGF production is accompanied by an increase in the expression of the VEGFR-1 on hepatocytes and of VEGFR-1 and VEGFR-2 on LSECs [67,73,93,97]. Activation of VEGFR-2 stimulates LSEC proliferation. On the other hand, binding of VEGF to VEGFR-1 on LSEC induces secretion of growth and survival factors such as HGF and interleukin-6 (IL-6), which in turn stimulate hepatocyte proliferation and reduce liver damage in mice exposed to CCl4 [96]. This suggests an angiogenesis-independent endothelial protection of hepatocytes through VEGFR-1.

In addition to VEGF and HGF, other angiogenic growth factors and their receptors are upregulated during liver regeneration, including PDGF, FGF and angiopoietins. Angiopoietins Ang1 and Ang2 regulate vessel stability by activating (Ang1) or antagonizing (Ang2) signalling via the receptor tyrosine kinase Tie2 [16]. Ang1 stabilizes vessels by promoting pericyte recruitment. Tie2 which is expressed by LSECs and its ligands Ang1 and Ang2 increases during liver regeneration [67,73,75,98]. The orphan receptor Tie1 was found to be expressed on LSECs surrounding avascular hepatocyte clusters [67]. Neuropilin-1 and Neuropilin-2 are recently discovered VEGF co-receptors unrelated to VEGFR-1 and -2, which have no intrinsic signalling but enhance binding of VEGF to VEGFR-2 [99,100]. Neuropilin-1 has been shown to be upregulated regenerating livers [98,101].

Thrombocytes play an important role in liver regeneration [102]. Thrombocytes contain high concentra-

tions of angiogenesis stimulators (i.e. VEGF, bFGF, PDGF) as well as inhibitors (i.e. endostatin, thrombospondin 1) packaged in distinct populations of α-granules which can be released selectively [74,103]. Although unexplored, one can speculate that as thrombocytes adhere to activated endothelium their action can enhance or inhibit local angiogenesis and thereby influence liver regeneration.

In summary, intense research using partial hepatectomy in animal models leads to identification of mechanisms and pathways which regulate angiogenesis in the liver. Emerging antiangiogenic drugs are under investigation to counterbalance dysregulated angiogenic pathways in chronic liver disease and HCC and are discussed in the other sections.

5. Angiogenesis and liver cancer

The growing incidence of hepatocellular carcinoma (HCC) on a worldwide scale [104] and the current capacity to diagnose and treat this cancer at an early stage has raised interest in this neoplasm [105,106]. It is well known that in most patients, this malignancy emerges in a liver with long-standing cell damage that has resulted in extensive fibrosis or cirrhosis [106]. In fact, HCC development is now a leading cause of death in cirrhotic patients. This has prompted major research activity into all aspects related to the pathogenesis and clinical translation of laboratory data into clinical practice.

There are several in-depth reviews that expand into the genomic abnormalities that might be found in experimental liver tumours as well as in cell lines and human tumour tissue banks [107–110]. An enormous amount of data describing chromosome abnormalities and gene expression has been published in recent years and gene expression has also been tentatively correlated with prognosis both with and without treatment application. Furthermore, analysis of tumours obtained at different evolutionary stages have allowed to propose gene events that herald the transformation of premalignant clones into overt HCC [111] and at the same time, the recognition of different gene expression patterns have triggered the proposal of a molecular classification of HCC [112–114]. Furthermore, the dissection of the derangement of several signal transduction pathways that govern cell proliferation, invasion and survival [115] has fuelled the development of new agents aimed at targeting the specific event that is responsible for cancer evolution [116]. Blockade of the effective signalling by growth factors has been generated in the benches and some of them have reached human testing. The same applies for the pathways that are abrogated and permit malignant cell survival, and also for the different molecules that are involved in the active neoangiogenic process that characterize all cancer types [117].

It is important to note that HCC is a highly vascularised tumour. Hence, profiling and targeting of the steps leading from a premalignant poorly vascularised nodule to the transition into an overt malignant phenotype with enhanced arterial blood supply has become a landmark event both for diagnosis, current treatment and novel therapeutic approaches [117,118].

5.1. Clinical relevance of the vascularization profile in HCC

The cell origin of HCC is not well-established. It may derive from hepatic stem cells or from the transformation from dysplastic hepatocytes into malignant cells. This last evolution has been quite well characterized in recent years [119] when imaging techniques have permitted the monitoring of the evolution from a small (<1 cm) hypoechoic nodule within a cirrhotic liver into typical HCC [120]. Small hypoechoic nodules have been given different names that include adenomatous hyperplasia, dysplastic nodule, macroregenerative nodule with dysplastic changes, but recently have been divided into regenerative nodules and low or high-grade dysplastic nodules [121,122]. The risk of malignant transformation of the first type is nil, it is minimal in the low-grade category and is really high in the nodules classified as high-grade dysplastic nodules [122]. All of them have a blood supply dependent from the portal vein, the hepatic artery supply being absent [123]. Because of this characteristic, the nodules are recognised as hypoechoic at plain ultrasonography and characteristically lack contrast uptake in the arterial phase if explored by contrast enhanced-US, dynamic CT or MRI [124]. In some cases the progressive transition into early HCC is associated with fat accumulation that turns the nodule into hyperechoic. If the nodule is first detected at that stage, it might be wrongly suspected to correspond to a small angioma, while it indeed corresponds to a transformed clone that it is likely to have experienced oxidative stress due to impaired blood supply. If stress is excessive some apoptotic death will take place, but ultimately, the malignant profile is established and overt cancer is in place. At this point, the vessel pattern has sharply changed and arterial supply is predominant. This feature is the basis for the imaging diagnosis of HCC within a cirrhotic liver [118,124]. Confident diagnosis is easy in large HCC as the vascular bed is fully established. However, at an early stage the arterial net is not extensive enough and only one-third of the nodules ultimately corresponding to an HCC can be confidently diagnosed by imaging characterization, the rest requiring a diagnostic biopsy.

Recent studies have tried to correlate gene expression in tumour tissue with imaging characterization [125].

Encouraging results have been reported but it is expected that a more sensitive assessment will be obtained through metabolic/molecular characterization by MRI, rather than with the mere evaluation of contrast behaviour. In that sense, the clinical need and urgent research challenge is not to classify already advanced cancer, but to characterize small nodules. It is crucial to confidently establish if they have not yet reached the oncogenic capacity or if they are already entering the neoangiogenic stage that precedes malignant evolution.

Detailed pathology studies carried out by the team of M Kojiro in Kurume, Japan have further divided small HCC into two different types: distinctly nodular vs. vaguely nodular [123]. The latter is assumed to correspond to the carcinoma *in situ* entity and lacks any invasive feature. By contrast, the distinctly nodular HCC type is clearly more evolved. A surrounding fibrotic capsule is more frequently observed and it exhibits a higher rate of microscopic vascular invasion. Interestingly, the transition from carcinoma *in situ* to established cancer is associated with the appearance of the prominent arterial blood supply that might be recognised by dynamic imaging techniques. Immunohistochemical staining is able to capture this neovessel formation and thus, staining for endothelial cells to display isolated arteries within a nodule is used as a diagnostic characteristic by pathologists. Hence, new blood vessel formation by either angiogenesis or vasculogenesis is a critical step not only for cancer progression as it occurs with all cancer types, but is recognised as the event that marks the frontier between the potential to achieve complete cure [126] and the stage where removal is feasible but the risk of dissemination is already acquired and long-term disease-free status is less likely.

The relevance of angiogenesis as a key event in the emergence and recognition of early HCC does not detract the relevance of intense arterial blood supply both for the development of recurrence after potentially curative therapy and also for the progression of advanced cancer. Growth of metastatic nests requires an extensive net of new arterial vessels to be formed and not surprisingly, progression from early to advanced cancer also requires this activity. Manoeuvres like chemoembolization employed to impair blood supply and induce ischemic necrosis have been one of the effective treatments for advanced HCC. Despite being in place for years, it has just been confirmed recently that data from randomised controlled trials have demonstrated that chemoembolization (a combination of arterial obstruction and selective chemotherapy administration) improves survival of patients that have reached this evolutionary stage [127]. Hence, acting against blood supply has become a validated target and thus the backbone of treatment of HCC diagnosed at an intermediate stage.

The relevance of vascularization in the evolution of HCC is also reinforced by several studies that have correlated the intensity of arterial blood supply or vessel formation inside the tumour with prognosis [128]. The same applies for markers of angiogenic activity in peripheral blood. Increased concentration of VEGF in serum has been correlated with outcome after surgical resection [129] or ablation [130]. Interestingly, HCC cell lines (Fig. 5) derived from human tumours may produce VEGF by themselves and this demonstrates their pivotal role in inducing vessel development [131].

All these data have provided the rationale to develop molecular targeted therapies that could provide an effective (biology) based treatment of HCC.

5.2. Targeted anti-angiogenic therapy

For years the treatment of HCC has been largely based on local procedures. Effective options have comprised blades, needles and devices aimed at removing or necrosing tumor cells. However, since cancer is a process governed by biologic events, the optimal approach should target the signals and events that make a malignant cell proliferate, invade, disseminate and survive. Angiogenesis and vasculogenesis are complex processes that involve many factors, the best characterized are fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and angiopoietins [132,133]. Recently, other factors such are semaphorin, ephrins [134], TGF-β/BMP [135] and Notch/Delta have been added to the list [136,137].

The VEGF growth factors are a family of homodimeric glycoproteins that are encoded by 4 VEGF genes (A–D), and a related growth factor, placental growth factor (PLGF) encoded by placenta growth factor gene PlGF [138]. VEGF-A gene produces five isoforms of VEGF-A protein, being that of 164 amino acids the isoform acting as the strongest mitogen. It is well known that VEGF-A expression is regulated by hypoxia, glucose concentration, pH and several oncogenes and all VEGF family members elicit their biological function

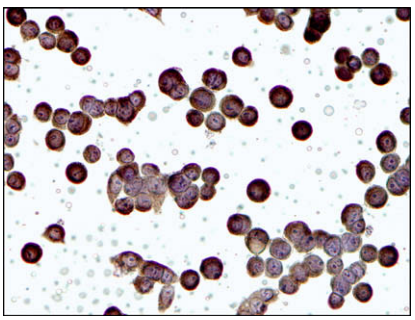

Fig. 5. BCLC-9 cell line derived from a human HCC. It has been shown to produce VEGF that is further enhanced under hypoxic conditions [131].

by their binding to cell-surface VEGF receptors (VEGF-Rs). The binding initiates a cellular response via activation of the intracellular tyrosine kinase domain of the receptor. VEGF-R2 (also called KDR) is the key receptor that induces both angiogenesis and vascular permeability [139].

Inhibitors of the VEGF pathway are the best characterized and consequently, the most clinically advanced agents to inhibit tumour angiogenesis.

At present, these include:

(a) monoclonal antibodies targeting VEGF-A or the VEGF receptors (mAb)
(b) a variety of small specific molecule that inhibit ligand-dependent receptor autophosphorylation of VEGF-R1 and VEGF-R2 (TKI)
(c) antisense and siRNA targeting VEGF-A or its receptors
(d) targeting microRNAs can block endothelial cell migration, proliferation and angiogenesis [140].

VEGF inhibitors induce the arrest of endothelial cell proliferation, regression of the existing vessels (increasing endothelial cell death), suppress the mobilization of endothelial progenitor cells from bone marrow (vasculogenesis), and are reported to be cytotoxic for some malignant cells.

Table 2 lists some agents that have undergone intensive investigation. For most, it is assumed that the mechanism of action is highly selective, but probably there are other unknown targets that are affected at the same time. It is likely that some agents that appear to be effective are so because of a combination of biological actions, while agents that would appear similar because they share the main target, may be useless or deleterious as a result of the additional molecules that may be affected. In that sense, clinical studies using VEGF inhibitors have provided a lot of relevant information but also, a disparity of results. The increasing evidence of the potential roles of VEGF in other cell types different than those implying the vasculature, make the side effects of anti-VEGF therapies a matter of concern [141].

Since safety of agents does not appear to be homogeneous careful adjustment of dosage is needed (a relevant aspect in patients with liver failure) or consider the extra-VEGF activity of each. In that sense, the safety profile and the phase 2 data available with sorafenib [142] allowed to conduct a phase 3 placebo controlled randomised clinical trial in patients with advanced HCC. The results have been unequivocal. Sorafenib delays tumour progression as evaluated by conventional radiology criteria and this translates into a highly significant improvement in survival [143]. Hence, nowadays sorafenib should be considered the first-line systemic therapy for patients with advanced HCC. Until now, these patients had no therapeutic option with established efficacy and thus, this is a landmark outcome. However, in addition to the benefits of an effective therapy for the patients with this malignancy, the positive findings of sorafenib have major consequences. They validate antiangiogenics as a useful therapeutic tool and at the same time, demonstrate that the search and hope for molecular targeted agents is viable and effective. Accordingly, clinical trials have to incorporate bullets for additional pathways and/or combine these new drugs with the one that has been shown to be effective. Sorafenib is not only an inhibitor of the receptor of vascular endothelial growth factor receptors (VEGFR1-3) but also of the platelet-derived growth factor receptor-beta (PDGFR-β), and of the Raf-1 and B-Raf pathway [144]. Raf kinases are MAPK kinases, key components of pro-survival pathway [145]. Raf-1 inhibits two pro-apoptotic kinases both of them implicated in oxidative stress-induced injury. c-Kit, the receptor for stem cell

Table 2
Some of the drugs that have shown anti-angiogenic activity and that have been investigated in experimental models and/or in humans.

Agent	Class	Target(s)
Bevacizumab (Avastin)	mAb	VEGF-A
Imatinib (Gleevec)	TKI	ABL1/2, PDGFR a/b, c-Kit
Dasatinib (Sprycel)	TKI	ABL1/2, PDGFR a/b, c-Kit, Src family
Nilotinib (Tasigna)	TKI	ABL1/2, PDGFR a/b, c-Kit
Sunitinib (Sutent)	TKI	VEGFR1-3,c-Kit,PDGFRa/b,RET,CSF1R, FLT3
Sorafenib (Nexavar)	TKI	VEGFR2,PDGFRb,c-Kit,FLT3,RAF1,BRAF
AG-013736 (Pfizer)		VEGFR, PDGFR
AMG706 (Amgen)		VEGFR,PDGFR,c-Kit, RET
AP23573 (Ariad Pharmaceuticals)		mTOR, VEGFR
AZD2171 (AstraZeneca)		VEGFR1-3, PDGFR
CCI-779 (Wyeth)		mTOR, VEGFR
CDP-791 (Imclone Systems)		VEGFR2
Everolimus (Novartis)		mTOR, VEGFR
XL184 (Exelixis)		MMET,VEGFR,RTK,FLT3,TIE2
XL880 (Exelixis)		c-Met, RTK
XL999 (Exelixis)		VEGFR,PDGFR, EGFR,FLT3, Src

factor, is also inhibited by sorafenib. The role of this receptor in normal tissues is to ensure the mobilization of endothelial progenitor cells to the sites of injury. Thus, c-Kit inhibition could provide a means of preventing vasculogenesis in tumours [146]. c-Kit and other elements are also inhibited at a lower intensity by sorafenib, and this collateral profile is a key characteristic to be taken into account when thinking of potential combinations as while efficacy may be increased as expected, the side effects may also be increased. This is especially important if it is recalled that in most HCC there is an underlying liver disease that should not be further aggravated by the therapy for HCC.

5.3. Summary and future perspectives

There is overwhelming evidence that angiogenesis is of paramount relevance in the field of liver cancer. It is key in all evolutionary stages of the disease and its evaluation is important for diagnosis and prognostic evaluation. At the same time, vessel development and function has become a therapeutic target for which there are agents that have shown therapeutic efficacy and impact in survival.

The advent of sorafenib for advanced HCC is an excellent example of the potential of research moving from bench to bedside, not only because of the benefit for patients, but also importantly since it confirms the expectations placed in molecular targeted therapies. Earlier stages of the disease may also benefit from sorafenib either as an adjuvant or even at preventing transition from premalignant to malignant stage. Obviously, combination of agents to increase the efficacy offered by sorafenib as a single agent is an easy to propose aim. However, a note of caution has to be raised, since combination of agents requires a careful preclinical evaluation of the mix and the conduction of phase 1-2 exploratory trials to define dosage and safety prior to going for efficacy, as well as a better knowledge of molecular pathophysiology.

As in anything in life, expertise and caution permit advancements, while the opposite may put patients at risk and misuse valuable resources. Collaborative teams including cell biologists and physicians should be encouraged. Only a candid and honest exchange of concepts and data among these multidisciplinary consortia which in turn will allow the proper design of effective research plans, based on our current knowledge will expand and permit a better future for patients with HCC.

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