Histol Histopathol (2009) 24: 1323-1341

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Angiogenesis and liver fibrogenesis

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Summary. Angiogenesis is a dynamic, hypoxia-stimulated and growth factor-dependent process, eventually leading to the formation of new vessels from pre-existing blood vessels. In the last decade experimental and clinical studies have described the occurrence of hepatic angiogenesis in a number of different pathophysiological conditions, including those involving inflammatory, fibrotic and ischemic features. In particular, the literature evidence indicates that hepatic angiogenesis is strictly associated with, and may even favour fibrogenic progression of chronic inflammatory liver diseases of different aetiology. In this review, current "in vivo" and "in vitro" evidence supporting the potential pathogenetic role of angiogenesis in chronic liver diseases will be reviewed in an attempt to outline cellular and molecular mechanisms involved, with a specific emphasis on the crucial role of hypoxic conditions and hepatic stellate cells (HSCs), particularly when activated to the myofibroblast-like pro-fibrogenic phenotype.

Key words: Liver angiogenesis, Hepatic stellate cells, Hepatic myofibroblasts, VEGF, Pro-angiogenic cytokines

1. Blood vessel formation and remodelling: introductory remarks and the aim of the review

Blood vessels have a fundamental role in delivering oxygen, nutrients and several biologically active molecules, as well as blood and cells of the immune system, to all tissues, and indeed constitute the first organ during embryo development and form the largest network in the human body (Carmeliet, 2003; Jain, 2003). Small and/or nascent blood vessels are simply composed of endothelial cells (ECs) whereas the walls of the largest and mature blood vessels are formed by ECs and mural cells, which are embedded in an extracellular matrix (ECM), with "mural cells" being a general definition usually including pericytes (as in medium-sized vessels) and smooth muscle cells (SMCs, as in the largest vessels).

Blood vessels can be formed and grow by means of several major processes that the current literature refers to as vasculogenesis, angiogenesis, arteriogenesis and collateral vessel growth (Carmeliet, 2003, 2004).

Vasculogenesis

This term has been used for a long time simply to denote "de novo" blood vessel formation during embryogenesis, in which angiogenic progenitor cells (hemangioblasts present in the different tissues/organs) are able to migrate to the sites of vascularization where they differentiate into ECs and start to form the initial vascular plexus (Carmeliet, 2003). In recent years several laboratories have shown that in addition to this "prenatal vasculogenesis", ECs can be formed in the adult (postnatal vasculogenesis) by endothelial progenitor cells (EPCs), mesangioblasts, multipotent adult progenitor cells or so-called side-population cells. Moreover, endothelial progenitors are likely to significantly contribute to EC formation in adult life, particularly in the presence of ischemic, malignant or chronic inflammatory conditions.

Angiogenesis

This process is usually referred to as a dynamic, hypoxia-stimulated and growth factor dependent process, eventually leading to the formation of new vessels from pre-existing blood vessels. Formation of new vessels by the process of angiogenesis can occur virtually in almost all tissues and organs and is considered a critical step for tissue repair and growth in

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several pathophysiological conditions (Yancopoulos et al., 2000; Carmeliet, 2003; Ferrara et al., 2003; Jain, 2003; Pugh and Ratcliffe, 2003). Although from an historical point of view angiogenesis has been originally implicated mainly in cancer, arthritis and psoriasis, as well as in many other diseases, today several laboratories suggest that pathological angiogenesis, then the formation of new vessels occurring during clinical conditions characterized by persisting inflammation, chronic wound healing and fibrogenesis, may provide a key contribution to disease progression (Carmeliet, 2003, 2004).

Arteriogenesis

This term refers to a process consisting of the remodelling of existing blood vessels in which ECs have already been differentiated into arterial ECs to increase luminal diameter in response to increased blood flow, a process that usually requires recruitment of SMCs.

Collateral vessel growth

This definition is employed to identify a process characterized by the expansive growth of pre-existing vessels that are then able to form collateral bridges between arterial networks.

The aim of the present review is to first offer a brief overview of the major basic mechanisms and events in angiogenesis, followed by an analysis of those cellular and tissue peculiarities that are likely to make hepatic angiogenesis significantly differ from homologous processes in other tissues or organs. The bulk of the review will be focussed on critically analysing all most recent results suggesting that, irrespective of aetiology, hepatic angiogenesis is likely to favour fibrogenic progression of chronic liver diseases (CLDs), with liver myofibroblasts (MFs) playing a crucial role, being able to act as pro-angiogenic cells, as well as cellular targets of pro-angiogenic cytokines and growth factors. Physiological angiogenesis (i.e., angiogenesis occurring after partial hepatectomy) will be shortly analysed, whereas the crucial relationships between angiogenesis and hepatocellular carcinoma or metastatic liver cancers, which for their relevance should deserve an independent analysis, will not be mentioned here. Interested readers may refer to recently published more comprehensive reviews (Medina et al., 2004; Lee et al., 2007; Kerbel, 2008).

2. Hypoxia as a major stimulus for angiogenesis

Hypoxia can be simply defined as an oxygenation state that is below the norm for a particular tissue. Several studies have pointed out that in the human body the average tissue partial pressure of oxygen (pO_2) is usually higher than 20 mm of Hg and that true hypoxic conditions may exist when pO_2 is below the limit of 10 mm of Hg (Vaupel et al., 2001; Dewhirst et al., 2008). By definition, cells react to the reduced levels of pO_2 by involving critical molecular mediators belonging to the family of hypoxia-inducible factors (HIFs) that facilitate oxygen delivery and adaptation to decreased oxygen levels by up-regulating several genes carrying the so-called hypoxia response elements (HRE) sequences in their promoter or enhancer. Sufficient to this review (the interested reader can refer to more detailed and comprehensive reviews by Semenza, 2001, 2004; Rankin and Giaccia, 2008) are the following major concepts.

1) At present three HIFs have been characterized (HIF-1, -2 and -3) which are all heterodimers formed by an oxygen sensitive and inducible HIF-α subunit and a constitutive, oxygen-independent, HIF-ß subunit (the latter known also as arylhydrocarbon receptor nuclear translocator or ARNT). The best characterized member of this family of transcription factors is HIF-1, which is formed by HIF-1α and HIF-1ß or ARNT.

2) Under normoxic conditions, HIF-1α is continuously modified by a number of enzymes (hydroxyl-proline hydroxylases or HPHs; asparaginyl hydroxylase FIH1) whose activity is dependent on oxygen levels. Two major mechanisms are known that prevent the formation of transcriptional complexes able to translocate into the nuclei and bind HRE sequences: a) HIF-1α, when hydroxylated on proline residues and/or acetylated on lysine residue in normoxic conditions, binds to the von Hippel-Lindau protein and other polypeptides forming a multi-subunit complex that is ubiquitylated and continuously degraded via proteasomes; b) alternatively, FIH1 can hydroxylate an asparagine residue of HIF-1α.

3) Under hypoxic conditions HPHs and/or FIH1 are progressively inhibited and HIF-1α can form the heterodimer with ARNT; HIF1 is then phosphorylated/stabilized by intervention of kinases and can form the ultimate transcriptional complex able to bind HRE sequences.

4) Sets of genes that are activated in a HIF-1-dependent manner include those involved in: a) vasomotor control and erithropoiesis, such as inducible NO synthase, endothelin 1 and erythropoietin; b) energy metabolism, such as glucose transporters (mainly GLUT-1 and -3) and several glycolytic enzymes; c) cell survival, including a number of ion transporters and exchangers able to regulate intracellular pH; d) cell proliferation and cell cycle; e) angiogenesis and ECM degradation and remodeling, including members of the VEGF family and related receptors, angiopoietins and related receptors, collagen prolyl-4-hydroxylase, the receptor for urokinase-plasminogen activator or uPA-R and the plasminogen activator inhibitor type 1 (PAI-1), to name just a few.

Relevant to this review, two more concepts should be recalled. First, it should be remembered that there are tissues or tissue areas in which pO_2 is low even in normal conditions: this occurs in the normal liver where the first rim of perivenular hepatocytes has been described to be under conditions of partial hypoxia, as

confirmed by the fact that these cells are positive to immunohistochemistry for HIF-1 (nuclei) and VEGF (cytoplasm), as well as for pimonidazole adducts (Arteel et al., 1995; Rosmorduc et al., 1999; Bozova and Elpek, 2007; Dewhirst et al., 2008). Second (Dewhirst et al., 2008), one should always remember that although under hypoxia HIF-1α activation is mostly obtained by post-translational mechanisms, there are conditions that are able either to lead to increased HIF-1α mRNA transcription (including cytokines, growth factors, oncogenes, metabolic stress and reactive oxygen species or ROS operating through activation of PI3K and Ras/Erk signalling pathways) or to HIF-1α increased stabilization (for example, following direct interaction with ROS). This scenario, in which HIF-1α may operate also independently on hypoxic conditions, is likely to play a role in conditions of CLDs.

3. Angiogenesis: basic mechanisms and events

Angiogenesis is a common event in CLDs (Medina et al., 2004) and is likely to be mostly dependent on the development of hypoxic conditions and of cellular responses mediated by the hypoxia-inducible factors. HIFs upregulate several angiogenic genes (Fig. 1) but the induction of vascular endothelial growth factor (VEGF) is perhaps the most remarkable one, and its increased transcription is rapid and impressive (up to 30 fold, depending on the specific cell type). VEGF can sustain both physiological and pathological angiogenesis, whereas other members of the VEGF family may have a role only in pathological angiogenesis, like placental growth factor (PlGF). Several other molecules are able to regulate angiogenesis, including growth factors, cytokines, chemokines, lipid mediators, hormones, neuropeptides and, possibly, also reactive oxygen species (ROS). A detailed analysis of cellular and molecular mechanisms controlling angiogenesis is beyond the scope of the present review and here only major general steps and events of the process will be schematically recalled (Carmeliet, 2003; Ferrara et al., 2003; Pugh and Ratcliffe, 2003; Medina et al., 2004; Dewhirst et al., 2008).

3.1 Sprouting and budding

If one considers quiescent vessels as the starting condition and hypoxia as the major initiating stimulus for angiogenesis, the first events are those depending on the action of nitric oxide (NO) and VEGF that can lead ECs to migrate and proliferate in order to allow new blood vessels to grow and branch. As depicted in Figure 2A, endothelial budding requires NO-dependent vasodilation and VEGF-induced increased vascular permeability with loosening of all those inter-endothelial contacts that in quiescent vessels provide mechanical strength and tightness and establish a permeability barrier. This includes mainly vascular endothelial cadherin in adherens junctions and claudins, occludins and junctional adhesion molecules (JAMs) in tight junctions, but also relevant are contacts through CD31

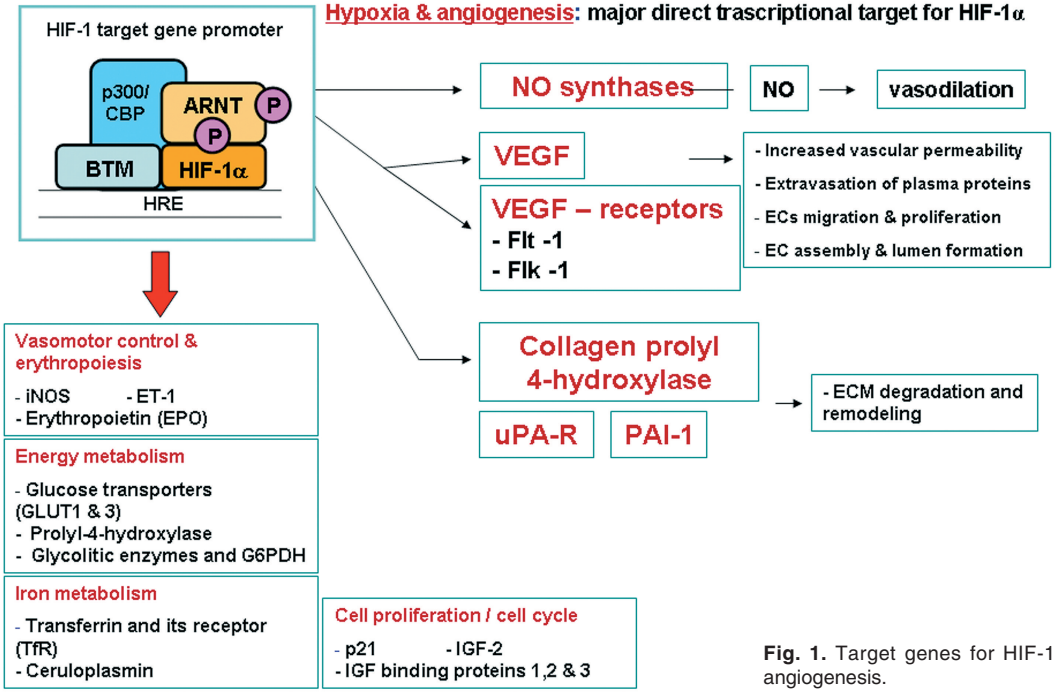

Fig. 1. Target genes for HIF-1 in mediating hypoxia-induced angiogenesis.

(PECAM1) and intercellular communications through connexins in gap junctions. Vasodilation and loosening of interendothelial contacts will result in leakiness of pre-existing vessels, leading to extravasation of plasma proteins that, together with extracellular matrix (ECM) components, will form a provisional scaffold or path for ECs to migrate. The main antagonist stimulus to these starting events in angiogenesis is represented by Angiopoietin-1 (Ang1), which tightens interendothelial contacts (Figs. 2A, 3).

3.2 ECM degradation and EC migration

In order to allow ECs to migrate, proliferate and then form new sprouts, the ECM network of pre-existing vessels (including basement membrane, as well as an interstitial matrix of elastin and collagen type I between vascular cells) has to be submitted to a carefully controlled and equilibrated process of proteolytic remodelling (Fig. 2B). ECM proteolytic remodelling is sustained by a number of proteinases and related inhibitors that include: a) matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), b) plasminogen activators (mainly urokinase plasminogen activator or uPA and its physiological inhibitor PAI-1), c) other proteinases, including heparinases and cathepsins. Proteinases can also contribute to EC migration by either liberating ECM-bound pro-angiogenic factors like VEGF, basic fibroblast growth factor (bFGF) and transforming growth factor ß1 (TGFß1), or by proteolitically activating other factors. Moreover, as a consequence of proteolytic remodelling, migration of ECs (and possibly pericytes and smooth muscle cells) may also be facilitated by exposure of cryptic epitopes in ECM proteins or by disruption of integrin-mediated contacts between ECs and ECM (Carmeliet, 2003). However, it should be mentioned that some integrins (αvß3 and αvß5) may also act as anti-angiogenic factors by inhibiting VEGF and VEGF receptor Type 2 (VEGFR-2 or Flk-1) mediated survival

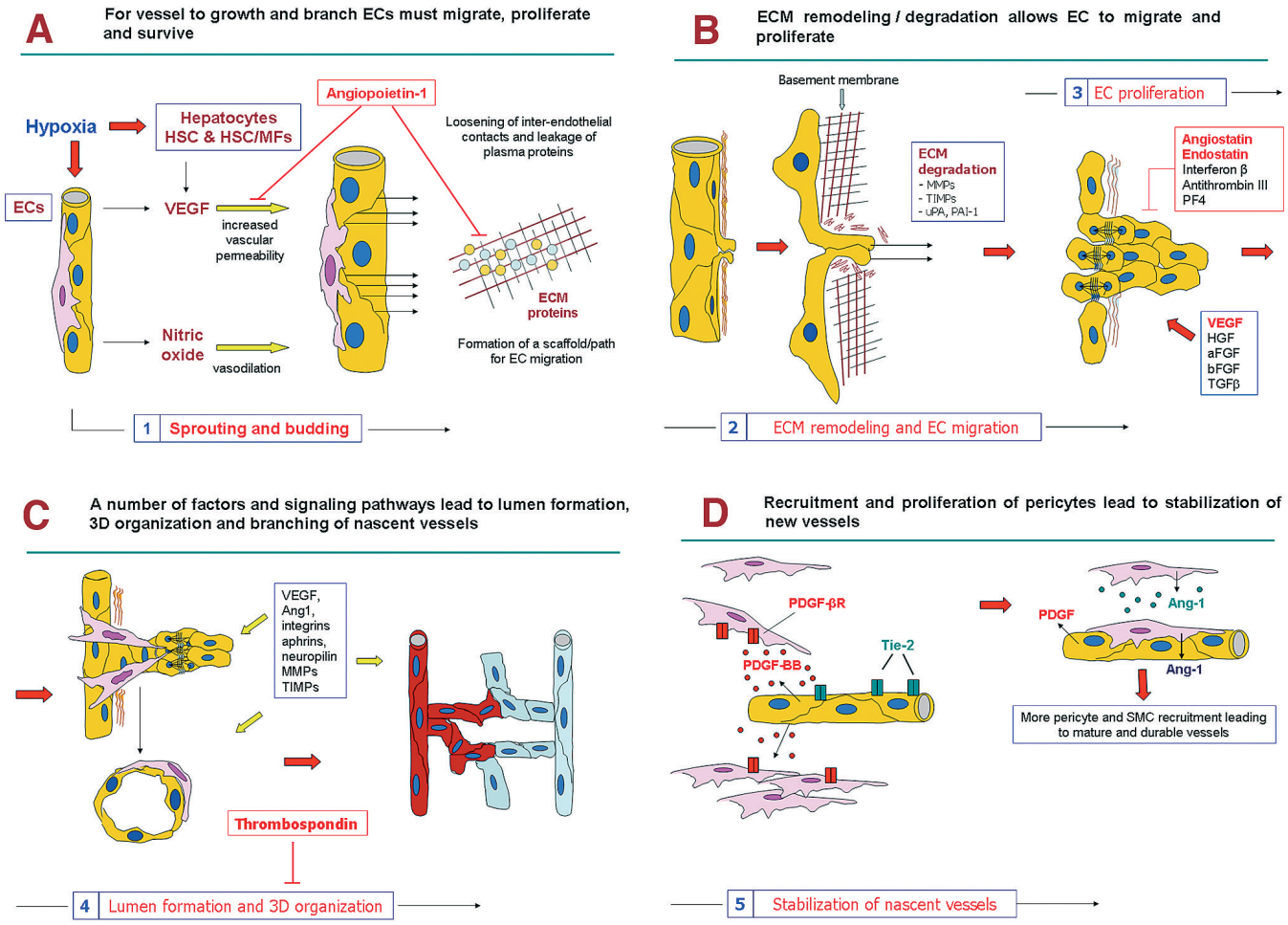

Fig. 2. Different stages of angiogenesis. **A.** Sprouting and budding due to endothelial cells (EC) proliferation and migration, mainly stimulated by hypoxia. **B.** Extracellular matrix (ECM) remodeling and EC migration and proliferation. **C.** Lumen formation and 3D organization. **D.** Stabilization of nascent vessels.

of ECs. The relevance of proteolytic remodelling for angiogenesis is confirmed by two opposite findings, both resulting in inhibition of angiogenesis (Luttun et al., 2000; Jackson, 2002): a) an excess of ECM proteolysis can result in the removal or destabilization of those critical epitopes able to guide migration of ECs; b) on the other hand, an insufficient or inadequate ECM proteolytic remodelling will again prevent migration of ECs.

3.3 EC proliferation, three dimensional organization and branching of new vessels

ECs will start then to proliferate in response to a number of mitogenic stimuli (see Figs. 2C, 3) including VEGF, basic and acidic FGF, hepatocyte growth factor (HGF) and TGFα and TGFß. The literature suggests that the most relevant stimulus is represented by VEGF released by ECs themselves, as well as by a number of neighbouring cells that for liver parenchyma after partial hepatectomy, or in CLDs, are represented by hepatocytes and hepatic stellate cells (HSC, which in normal liver have been described to behave also as liver specific pericytes), with kupffer cells and leukocytes possibly making an additional contribution (Medina et al., 2004). VEGF acts mainly on cells expressing VEGF type 1 (VEGFR-1) and type 2 (VEGFR-2) receptors (also known as Flt-1 and Flk-1, respectively). Additional positive stimuli for EC proliferation are provided by cytokines, certain chemokines (others may exert the opposite effect), hormones and lipid mediators whereas the list of negative mediators able to inhibit EC proliferation include interferon ß, antithrombin III, platelet derived factor 4, leukaemia inhibiting factor, with angiostatin and endostatin being the most effective peptides in suppressing ECs proliferation (Carmeliet, 2003).

Once EC proliferation is switched on in an ordered manner, other steps, signalling pathways and mediators are required for three-dimensional organization of nascent vessels and for their conversion into mature vessels in order to establish a functional vascular network (Fig. 2C,D). The first step is represented by the formation of a lumen in the nascent vessel that, together with its diameter and length, is mainly affected by the action of VEGF, Ang1 and integrins αvß3 and αvß5, whereas thrombospondin has been suggested to act as a major antagonist of this angiogenic step. Three dimensional organization of an efficient vascular network of vessels of uniform size additionally requires a carefully orchestrated intervention of mediators and activation of signalling pathways, resulting again in cell proliferation and migration, as well as in the organized branching of new vessels (mainly regulated by ephrins and neuropilins) and in MMPs- and TIMPs-regulated deposition of ECM components and formation of basement membrane.

3.4 Vessel maintenance, growth and stabilization versus vessel regression

Conversion of nascent vessels into mature and stabilized vessels now requires the progressive association of pericytes or pericyte-like cells to newly

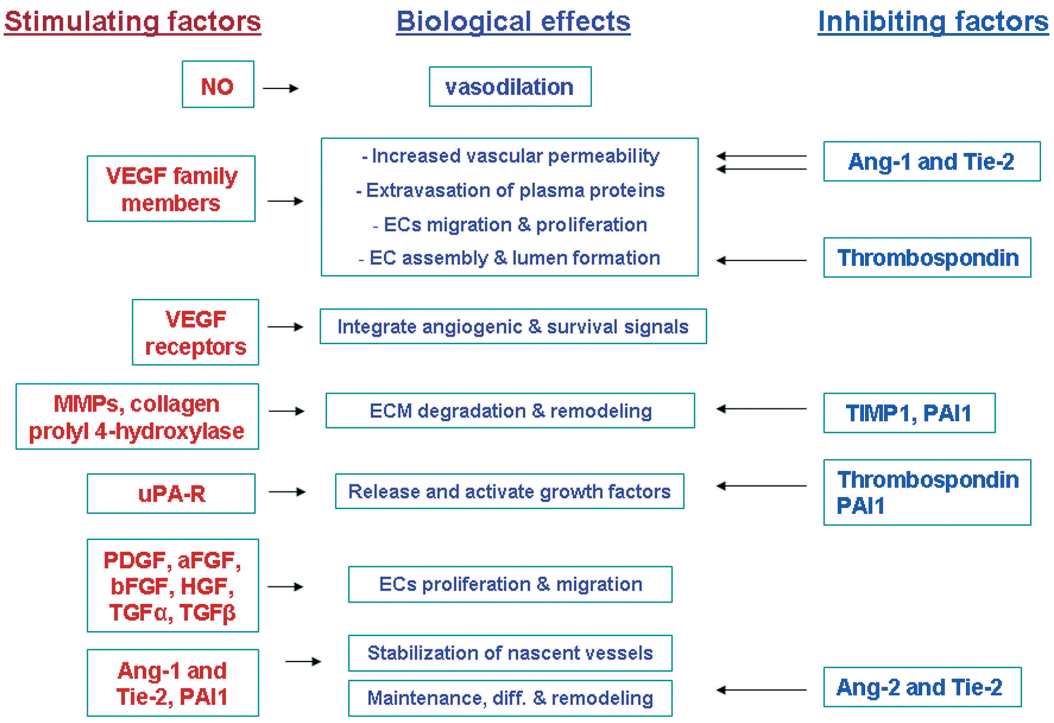

Fig. 3. Angiogenesis as a dynamic process: major molecules involved and their role.

formed vessels (Carmeliet, 2003; Jain, 2003), a crucial event that also offers a significant contribution in regulating EC proliferation, migration, survival, differentiation, vascular branching, blood flow and vascular permeability. In this setting a crucial role in the stabilization of nascent vessels is provided by platelet-derived growth factor (PDGF)-BB and its related receptor ß-subunit (PDGFR-ß). PDGF-BB, mainly released by ECs, contributes to recruit PDGFR-ß positive mesenchymal cells or progenitors to nascent vessels, leading also to their proliferation (Fig. 2D). Lack of recruitment of mural cells to nascent vessels can deeply affect vessel formation, resulting in more fragile, larger and permeable vessels and then in bleeding, decreased perfusion and hypoxia (Hellström et al., 2001; Jain, 2003). Once recruited to nascent vessels mesenchymal cells or progenitors can be differentiated into pericytes by TGFß1, which is also fundamental in sustaining deposition of ECM components. However, the most important scenario for vessel stabilization is represented by interactions between pericytes and ECs of nascent vessels. Recruited pericytes start to release Ang1 that interacts with the corresponding receptor Tie-2 expressed on ECs, an event that tightens vessels by affecting junctional molecules and by further promoting interactions between ECs and mural cells (Jain, 2003), possibly acting as a molecule facilitating ECs adhesion mediated by integrins (Carlson et al., 2001). However, one should remember that an excess of Ang1 can result in the formation of too tightened vessels, preventing further sprouting and even blocking angiogenesis.

An interesting role is the one played by Ang2, which can activate Tie-2 in some cells and block it on others. Although some studies have proposed a pro-angiogenic role for Ang2 (for example in the heart by synergizing with VEGF), it is established that when a lack of pro-angiogenic signals occurs (including VEGF, PlGF, PDGF-BB, Ang1) and/or an excess of angiogenic inhibitors (thrombospondin, interferon ß and others) is present in the microenvironment, Ang2 may induce cell death of ECs and sustain vessel regression (Carmeliet, 2003, 2004).

4. Angiogenesis and the liver

Physiological and pathological angiogenesis have been clearly identified in the following conditions: a) during liver regeneration after acute liver injury or after partial hepatectomy; b) in ischemic conditions; c) during chronic inflammatory and fibrogenic liver diseases; d) in hepatocellular carcinoma and in metastatic liver cancers (Medina et al., 2004).

Hepatic angiogenesis proceeds with steps and molecular mechanisms that mostly overlap with those just described in the previous paragraph. However, as already pointed out by Medina et al. (2004), liver angiogenesis is likely to be significantly affected by a number of liver parenchyma peculiarities. First, the liver is a peculiar organ characterized by the existence of two different kinds of microvascular structures: a) large vessels lined by a continuous endothelium lying on a standard basement membrane, such as portal vessels (portal veins, hepatic arterioles) and centrilobular veins; b) liver sinusoids that are lined, in normal conditions, by fenestrated and discontinuous ECs. Second, Camenisch and coworkers (2002) have described the existence of a liver derived angiopoietin-like peptide defined as ANGPTL3; this peptide is unable to bind to Tie2 receptor but can bind αvß3 integrin, inducing haptotactic endothelial cell adhesion and migration and stimulated signal transduction pathways characteristic for integrin activation, including phosphorylation of Akt, activation of mitogen-activated protein kinase and focal adhesion kinase. However, although ANGPTL3 has been shown to operate as a potent pro-angiogenic peptide both “in vivo” and “in vitro”, no data are available at present on its role in either physiological or pathological liver angiogenesis.

Last, but not least, the unique and heterogenous phenotypic profile and functional role of hepatic stellate cells (HSCs): these peculiar cells are also regarded as liver specific pericytes in normal liver (Friedman, 2008a) but their role in modulating angiogenesis may differ from the role attributed to microcapillary pericytes (Lee et al., 2007). As will be emphasized later in this review, this is likely to occur mainly in pathological conditions, such as during fibrotic progression of CLDs, where myofibroblast-like cells (MFs) originating from activated HSC (HSC/MFs) play a major pro-fibrogenic role. The scenario is even more complex if one considers that hepatic MFs constitute a heterogenous population of pro-fibrogenic, highly proliferative and contractile cells that may also originate from portal (myo)fibroblasts, bone marrow-derived stem cells and, possibly, also from hepatocytes and or cholangiocytes through a process of epithelial to mesenchymal transition (Friedman, 2008b; Parola et al., 2008).

4.1 Physiological hepatic angiogenesis

Most reliable data have been obtained by studies designed to investigate revascularization after experimental partial hepatectomy (PH), which is considered the most appropriate model to investigate physiological hepatic angiogenesis. Hepatic physiological angiogenesis was well reviewed by Medina and coworkers some years ago (Medina et al., 2004 and references therein) and here only major specific events will be summarized as follows:

a) After two/third PH, the first relevant biologic event is represented by an immediate and strong proliferative response of parenchymal cells (usually with two peaks of DNA synthesis at approx. 24 and 48 hrs), mainly sustained by TGFα released by hepatocytes and HGF released by non-parenchymal cells; this early proliferative response involves mainly hepatocytes in the periportal areas and this is crucial since it leads to the formation in the same areas of avascular (i.e., hypoxic)

clusters of hepatocytes.

b) Hepatocytes of these periportal clusters respond to hypoxic conditions by up-regulating transcription of VEGF within 48-72 hrs from PH; VEGF is then released in a paracrine fashion and can act on all cells in which the expression of VEGF receptors type 1 and 2 has been detected, starting from 72 hrs and persisting for several days, including arteriolar and sinusoidal ECs as well as HSCs. Accordingly, reconstitution of sinusoids by ECs initiate then in the same periportal areas

c) VEGF, which may also concur to sustain hepatocyte proliferation, has also been reported to prime ECs to express a number of growth factors that may contribute to prevent hepatocyte injury, as well as to exert its effects (proliferation, synthesis of ECM) on HSCs. Finally, as will be recalled later, VEGF can also be released by HSCs exposed to hypoxic conditions.

5. Angiogenesis in chronic liver diseases characterized by persisting inflammation and progressive fibrogenesis: "in vivo" evidence

Chronic liver diseases are usually characterized by reiteration of liver injury due to a number of aetiological conditions, including chronic infection by viral agents (mainly by hepatitis B and C viruses) as well as metabolic, toxic/drug-induced (with alcohol consumption being predominant) and autoimmune causes. This is known to result in persisting inflammation and progressive fibrogenesis, with chronic activation of the wound healing response representing a major driving force for progressive accumulation of ECM components, eventually leading to liver cirrhosis and hepatic failure. Other mechanisms sustaining fibrogenic progression of CLDs include oxidative stress and redox signalling, derangement of epithelial - mesenchymal interactions and, as emerging evidence suggests, the process of epithelial to mesenchymal transition (Parola and Robino, 2001; Friedman, 2003, 2004, 2008b; Bataller and Brenner, 2005; Novo and Parola, 2008; Parola et al., 2008). Although different patterns of fibrosis progression have been reported to exist that may depend on the specific aetiology, as well as the prevailing pro-fibrogenic mechanism (Pinzani and Rombouts, 2004; Parola et al., 2008), cirrhosis is indeed currently envisaged as an advanced stage of fibrosis that is typically characterized by a common scenario which includes the formation of regenerative nodules of parenchyma surrounded and separated by fibrotic septa and associated with significant changes in angio-architecture.

As mentioned in the introductory remarks, it is a common feeling that angiogenesis as a process, when occurring during hepatic chronic wound healing and fibrogenesis, may significantly contribute to disease progression (Friedman, 2004, 2008b; Medina et al., 2004; Lee et al., 2007; Parola et al., 2008). This hypothesis of course relies first on the established concept that angiogenesis is a major process involved in wound healing, being integrated with the inflammatory process (Carmeliet, 2003). Moreover, it is well known that a common finding in human cirrhotic livers, irrespective of the aetiology, is represented by enhanced vascular remodelling (i.e., a condition that by itself suggests involvement of pathological angiogenesis). If angiogenesis in chronic inflammatory and fibrotic liver injury is concerned, one has also to remember that formation of fibrotic septa, as well as capillarization of sinusoids, the latter due to early deposition of fibrillar ECM in the space of Disse, can result in an increased resistance to blood flow and oxygen delivery (i.e., the premises for hypoxia), irrespective of aetiology. This is relevant since, as previously suggested, hypoxia is by far the main and most obvious stimulus for angiogenesis in any organ or tissue, with transcription of hypoxia-sensitive pro-angiogenic genes usually being modulated through HIFs.

In addition, from a general point of view, one has to emphasize the well known relationships between the inflammatory process and angiogenesis. Indeed, during the course of CLDs the inflammatory response gains the role of a dynamic state relevant for the progression of fibrogenesis towards the end-point of cirrhosis (Friedman, 2004, 2008b). If relationships between inflammation and angiogenesis are concerned, it is well known that several mediators of the inflammatory response may stimulate other cells in the surrounding microenvironment to express VEGF and other pro-angiogenic factors (Carmeliet, 2004). Other cytokines and mediators, known to be overexpressed during the condition of chronic inflammatory liver injury, have been suggested to play a role in the development of angiogenesis, including HGF, NO and PDGF (Medina et al., 2004). Moreover, one should consider that: a) neo-vessels are likely to significantly contribute to perpetuation of the inflammatory response by expressing chemokines and adhesion molecules promoting the recruitment or inflammatory cells; b) angiogenesis, early in the course of a CLD, may even contribute to the transition from acute to chronic inflammation (Jackson et al., 1997).

After these general concepts, we will analyze in the following sections the relationships between angiogenesis, inflammation and progressive fibrogenesis on the basis of most significant "in vivo" and "in vitro" published literature data and, where possible, offer "ready to use" overall messages.

5.1 Angiogenesis in clinical and experimental conditions of CLDs

A first message to be delivered is straightforward: unequivocal evidence of angiogenesis, including overexpression of pro-angiogenic cytokines and related receptors, has been detected in all relevant clinical conditions of CLDs, irrespective of aetiology, as well as in the most widely used experimental animal models of CLDs (Medina et al., 2004; Lee et al., 2007) and an

overview of available data on cellular source and/or localization of angiogenic molecules in CLDs is offered in Figure 4. Moreover, in most pathological conditions angiogenesis and fibrogenesis seem to develop in parallel during progression towards cirrhosis, although for obvious reasons the most detailed studies have been performed on experimental models (Rosmorduc et al., 1999; Corpechot et al., 2002a; Yoshiji et al., 2003; Kitade et al., 2006; Novo et al., 2007; Tugues et al., 2007; Taura et al., 2008; Moon et al., 2009; see also sections from 5.2 to 5.5). Apart from those events that may be considered to be in line with the general scheme of angiogenesis, a number of findings related mainly to chronic viral hepatitis and autoimmune diseases deserve further comment.

Chronic infection by HBV and HCV

Liver biopsies from patients affected by chronic viral hepatitis have shown the presence of ECs and neovessels in the form of capillary structures found in inflamed portal tracts (García-Monzón et al., 1995; Mazzanti et al., 1997; Medina et al., 2004). All major angiogenic molecules have been found to be over-expressed in these patients, including VEGF and HGF (Okajima et al., 1997; Okano et al., 1999; Shimoda et al., 1999; Medina et al., 2003a); PDGF has been found overexpressed in periportal inflammatory cells, sinusoidal and perisinusoidal cells (Pinzani et al., 1996; Ikura et al., 1997). If overexpression of VEGF and PDGF is likely to result in established pro-angiogenic effects for these growth factors, such as migration and proliferation of ECs for VEGF, or stabilization of nascent vessels for PDGF, HGF, which is a well known potent inducer of angiogenesis (Bussolino et al., 1992; Grant et al., 1993), has been described to stimulate proliferation and migration of ECs either by direct actions on ECs or through indirect paracrine stimulation of neighbouring cells such as SMCs to express VEGF (Van Belle et al., 1998; Medina et al., 2003a) .

Interestingly, it has been proposed for patients affected by chronic viral hepatitis that some selected viral proteins may have a pro-angiogenic role. A well characterized example is represented by HBV-related X protein, which has beenshown to be involved in disruption of inter-endothelial junctions by operating through a src-kinase-dependent signalling pathway (Lara-Pezzi et al., 2001), as well as in the up-regulation of inducible nitric oxide synthase (iNOS) through involvement of nuclear factor - κB (NF-κB) transcription factor (Majano et al., 2001). Indirect evidence also suggests that the same viral protein may be able to up-regulate in hepatocytes membrane-type MMP (MT-MMP) expression, and then MMP-2 activation, through mechanisms requiring cyclooxygenase 2 activation (Lara-Pezzi et al., 2002); this event may favour angiogenesis (see previous section 3.2) as well as hepatocyte invasion.

Another interesting finding (see also section 5.5 on the role of MFs in angiogenesis for more details) detected in biopsies from chronic HCV patients is represented by the fact that tiny and incomplete developing fibrotic septa show the existence, at their leading or lateral edge, of MF - like α-smooth muscle actin (α-SMA) positive cells that are also positive for VEGF, Ang-1 and Tie-2 (Novo et al., 2007). This scenario, which is homologous to the one detected in the cirrhotic rat livers following chronic CCl_4 treatment,

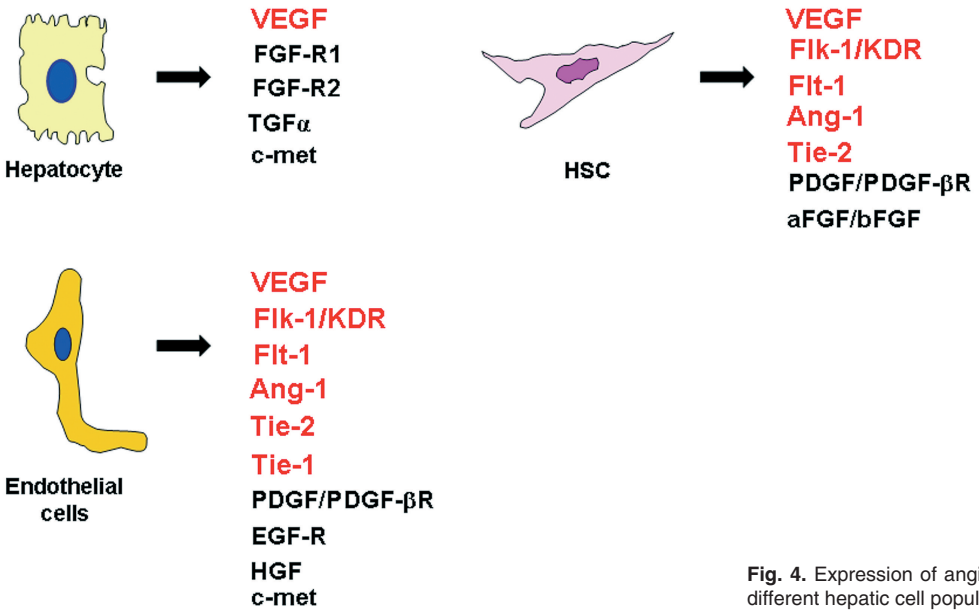

Fig. 4. Expression of angiogenic molecules and their receptors by different hepatic cell populations involved in chronic liver diseases.

consistently differs from images observed for larger and more mature septa, where α-SMA positive cells (i.e., MFs) are distinct from cells positive for VEGF (hepatocytes and ECs) or Flk-1 and Tie-2 (mainly ECs). As will be emphasized later, these findings may reflect two different phases of angiogenesis during chronic wound healing in chronic HCV patients.

Autoimmune chronic liver diseases

Evidence for angiogenesis was detected in biopsies from patients affected by either primary biliary cirrhosis (PBC) or autoimmune hepatitis as formation of tubule-like structures (i.e., neovessels) by ECs positive for CD-31 and vascular endothelial-cadherin (Medina et al., 2003b, 2004, 2005). These neovessels were located, particularly for PBC, mainly in portal areas in association with inflammatory infiltrate (Medina et al., 2005).

Where PBC is concerned, enhanced expression of angiogenic molecules like VEGF, Ang-1, Ang-2, Tie-2 and endoglin has been also characterized in PBC patients. Moreover, additional indirect evidence suggesting the existence of angiogenesis in PBC was in the report that ECs are positive for fibronectin and laminin (known to be involved in the regulation of angiogenesis) and, interestingly, that ECs of the peribiliary plexus are positive for ß1 integrins (Yasoshima et al., 2000).

It should be noted, in this connection, that some authors have reported for autoimmune diseases like PBC, primary sclerosing cholangitis (PSC) and autoimmune hepatitis, a tendency to develop vasopenia and a decrease in peribiliary capillary plexus (Washington et al., 1997; Matsunaga and Terada, 1999). This scenario was attributed to destruction of vascular structures by autoimmune mechanisms, similar to the process undergone by bile ducts in PBC and PSC; vessels (including newly formed capillaries) might also be destroyed during the scarring phases of the disease. However, as proposed by Medina and coworkers (Medina et al., 2004, 2005), this apparent controversy might be simply related to the different temporal dynamics of neoangiogenesis and autoimmune destruction of vascular structures in PBC.

5.2 Hypoxia and pathological angiogenesis in CLDs

Another major message offered mainly by experimental models of CLDs is that overexpression of the prototype pro-angiogenic cytokine VEGF is usually found in hypoxic areas, according to the rational hypothesis that hypoxia may represent the major stimulus for hepatic angiogenesis. Indeed, animal models offer the unique opportunity to use antibodies rised against pimonidazole adducts to identify hypoxic areas: pimonidazole and other related nitroimidazole derivatives have been described to bind macromolecules in cells exposed to low oxygen levels, and pimonidazole binding has been shown to be effective in assessing changes in hepatic tissue oxygenation (Arteel et al., 1995, 1997).

VEGF expression in normal liver, as well as its colocalization with pimonidazole adducts or nuclear HIF1α is mostly limited, as already mentioned in section 2, to a few hepatocytes constituting the first row around centrilobular vein (Arteel et al., 1995; Rosmorduc et al., 1999; Corpechot et al., 2002a; Gaudio et al., 2006; Bozova and Elpek, 2007; Tugues et al., 2007). An apparent exception to this rule has been reported for cholangiocytes that, at least in one study, seem to express VEGF even in normal conditions (Gaudio et al., 2006).

Historically, the first report of a close association between hypoxic areas, VEGF expression and then EC proliferation and angiogenesis in conditions of experimental CLDs was provided for the model of bile duct ligation (Rosmorduc et al., 1999). Most, if not all positive immunostaining for VEGF was detected in hepatocytes with a percentage of positive parenchymal cells ranging from 95% at 2 weeks after BDL to approx. 100% at the time of established biliary cirrhosis. In this study no apparent immunopositive stain for VEGF was detected in either hepatic stellate cells or cholangiocytes. This scenario differs from that reported by others concerning HSC and HSC/MFs for other conditions of liver injury and CLDs (see later), as well as from that described in a study using the same model of BDL (Gaudio et al., 2006) showing that cholangiocytes of the bile ductular reaction are able to express VEGF as well as VEGF receptors type 2 (Flk-1) and 3, suggesting a possible mitogenic effect for VEGF to sustain both proliferation of cholangiocyte (paracrine/autocrine effect) and ECs of microcapillary peribiliary plexus (paracrine effect).

The colocalization of hypoxic areas with VEGF overexpression and/or the association between VEGF expression and progression of fibrogenesis was then confirmed by the same group using the diethyl-nitrosamine (DEN) model of fibrosis (Corpechot et al., 2002a), as well as by others employing the model of CLD induced by chronic treatment with CCl_4 (Yoshiji et al., 2003; Novo et al., 2007; Tugues et al., 2007) or by feeding a choline-deficient and aminoacid-defined diet able to induce in rats a NAFLD, developing in time into NASH and significant fibrosis (Kitade et al., 2006). In most of these studies (see later sections 6.2 and 6.3 for more details) parallel "in vitro" and "in vivo" experiments were starting to outline that not only hepatocytes, but also HSC and/or HSC/MFs, particularly under hypoxic conditions, may be able to express both angiogenic cytokines as well as related receptors. Of relevance, in a study very recently published on-line (Moon et al., 2009), the strict relationships between hypoxia, angiogenesis, inflammation and fibrogenesis have been unequivocally confirmed by using liver conditional HIF-1α-deficient mice that were subjected to BDL: in these mice, where a very significant decrease in

collagen type I and α-SMA transcripts and protein levels, as well as of transcripts for PDGF and plasminogen activator inhibitor-1 (PAI-1) was detected, vs respective wild type mice in which the typical scenario of biliary type fibrosis and cirrhosis was associated to early and sustained up-regulation of HIF-1α.

Before leaving this section, another relevant study, able to further emphasize the role of hepatic hypoxia during the development of CLDs, should be at least mentioned (Corpechot et al., 2002b). Some years ago it was reported that hypoxia can negatively affect hyperplastic reponse of hepatocytes by at least two additional mechanisms: a) by down-regulating the ability of HSC to express HGF, the most potent mitogenic stimulus for parenchymal cells; b) by rapidly inhibiting c-met expression in hepatocytes. It is clear that these two mechanisms are likely to significantly contribute to depress liver regeneration during chronic liver injury.

5.3 Pathological angiogenesis as a potential therapeutic target

On the basis of concepts and data presented in the previous sections and chapters, an obvious consequence of the proposed relationships between angiogenesis and chronic wound healing and fibrogenesis (i.e., angiogenesis may contribute to disease progression) should include two main implications: a) a careful, non-invasive, detection of marker molecules involved in pathological angiogenesis may offer a way to monitor both disease progression as well as the response to the therapy; b) pathological angiogenesis may become a potential therapeutic target in patients affected by CLDs.

Concerning the first point, we are still far from having enough reliable data to be translated to clinically relevant conditions. To our knowledge just a single study on 36 patients affected by chronic hepatitis C (vs 15 healthy controls) has provided a serious attempt to correlate circulating levels of molecules involved in the angiogenic process with disease progression and efficacy of standard combination therapy based on pegylated interferon alfa-2b (IFN-α2b) and ribavirin (Salcedo et al., 2005). In this study serum levels of VEGF, Ang-2 and soluble Tie-2 (sTie-2) were determined before and after therapy and the authors reported that patients affected by chronic hepatitis C (CHC) showed elevated baseline VEGF and Ang-2 levels; moreover, after therapy both angiogenic factors were significantly decreased, whereas antiangiogenic sTie-2 was increased, results that were interpreted as evidence of a shift toward an "anti-angiogenic" profile of serum markers in CHC patients responding positively to the therapy.

As far as second implication is concerned, available data on experimental animal models of CLDs unequivocally indicate that antiangiogenic therapy is effective in preventing progressive fibrogenesis. The first study to deal with experimental antiangiogenic therapy for CLDs was based on "in vivo" administration of the semisynthetic analogue of fumagillin TNP-470 (Wang et al., 2000). This potent antiangiogenic inhibitor was used because of its very low toxicity (Ingber et al., 1990; Kusaka et al., 1991), the documented experimental ability to effectively inhibit growth of hepatocellular carcinoma (HCC) and of hepatic metastasis (Tanaka et al., 1995; Shishido et al., 1996; Ikebe et al., 1998) and, more relevant, the ability to also inhibit proliferation of mesangial cells and vascular SMCs (Haraguchi et al., 1997; Koyama et al., 1996). Repetitive subcutaneous injection of TNP-470 to rats submitted to either the CCl_4-or diethylnitrosamine-induced liver fibrosis resulted in a very significant reduction of ECM deposition. Data obtained for CCl_4-dependent chronic liver injury also indicated a sharp reduction in the number of α-SMA positive cells and of the bromodeoxyuridine (BrdU) positive cells. Indeed, in vitro experiments performed on primary culture of rat HSC showed that TNP-470 was able to inhibit proliferation of these cells by blocking cell cycle transition from G1 to S phase, as well as to inhibit their activation (Wang et al., 2000).

Another elegant and effective antiangiogenic approach was obtained by "in vivo" administration to BalbC mice, chronically treated with CCl_4, of antibodies able to neutralize either VEGFR-1 (Flt-1) and/or VEGFR-2 (Flk-1) (Yoshiji et al., 2003). Once again, this treatment was able to significantly inhibit angiogenesis, the number of α-SMA positive cells and the development of fibrosis. Two other major findings or concepts were provided by this study: a) VEGF expression (mainly the alternative mouse splicing VEGF genes leading to $VEGF_{120}$ and $VEGF_{164}$) was a prerequisite for murine fibrogenesis since neutralizing antibodies were administered after two weeks of treatment; b) although combination treatment with both antibodies was slightly more effective, the most relevant anti-fibrotic effect was by far observed using the anti-VEGFR-2 antibody, suggesting "in vivo" predominance of VEGF interaction with Flk-1 to mediate angiogenesis during chronic liver injury.

The latter finding was remarked by other observations provided by Fernandez and colleagues (Fernandez et al., 2004, 2005) who, by again using the antibodies able to neutralize VEGFR-2 in a model of portal hypertensive rats, were also able to correlate VEGF expression and related angiogenesis to the development of porto-systemic collateral vessels and of hyperdynamic splancnic circulation. These findings (see also Morales-Ruiz and Jimenez, 2005; Morales-Ruiz et al., 2005) are of intrinsic relevance because they suggest that the increase in portal blood flow, which is an important contributor to portal hypertension, depends not only on vasodilation, but also on the enlargement of the splancnic vascular tree caused by angiogenesis.

A more recent antiangiogenic approach (Tugues et al., 2007) has taken advantage of the multitargeted tyrosine kinase receptor inhibitor Sunitinib (SU11248). The rationale to use this indolinone derivative relied on

the fact that it was designed as a drug, having a broad selectivity for several receptor tyrosine kinases (Smith et al., 2004), with an already established antitumour and antiangiogenic effects in clinical trials for cancer treatment (Abrams et al., 2003; Deeks and Keating, 2006). In particular, the antiangiogenic effect of Sunitinib is attributable at least to inhibition of VEGF and PDGF receptors which, as reported in section 3, are both essential for angiogenesis (Carmeliet, 2003; Armulik et al., 2005). Moreover, it is well known that PDGF (particularly PDGF-BB and its related receptor ß subunit or PDGFR-ß) represent the most potent mitogenic and chemotactic agent for HSC and HSC/MFs (Pinzani et al., 1989; Pinzani and Marra, 2001; Friedman, 2008a,b). In the study by Tugues and coworkers, where cirrhosis progression in the liver of rats chronically treated with CCl4 was associated with an increased expression of VEGF, Ang-1, Ang-2 and PlGF, as well as of hepatic and splanchnic vascularization, the treatment of cirrhotic animals with Sunitinib resulted in a significant decrease of a number of inter-related pathological events, including hepatic vascular density, inflammatory infiltrate, abundance of α-SMA positive mesenchymal cells, ECM deposition and portal pressure. Parallel "in vitro" experiments performed on the human immortalized HSC cell line LX-2 were also able to show that Sunitinib was indeed affecting the response of these cells to PDGF-BB.

The experimental antiangiogenic approaches previously reported were all mainly directed at blocking the action of VEGF, widely accepted as the most potent pro-angiogenic cytokine in both physiological and pathological conditions. However, in the last two years "in vivo" studies from different laboratories have outlined that the proangiogenic role of HSC (see section 6.2 for more details) can also be mediated by the release of Ang-1, as seen in either experimental fibrotic and cirrhotic livers or in biopsies from HCV cirrhotic patients (Novo et al., 2007; Taura et al., 2008). Both laboratories reported that in cirrhotic livers immunostaining for Ang-1 was colocalized with α-SMA, a marker of myofibroblast-like cells. In experimental animals subjected to BDL or chronically treated with CCl_4 a parallel increase of CD31 and Ang-1 mRNA was reported, suggesting a significant angiogenic role of Ang-1 (Taura et al., 2008). This hypothesis was confirmed by the same group by injecting mice under BDL or chronic CCl_4 treatment with an adenovirus expressing soluble Tie-2 (AdsTie-2, the receptor for Ang-1, then able to block angiopoietin signalling), a procedure that resulted in a significant prevention of both angiogenesis and fibrosis.

5.4 The "in vivo" role of other mediators on angiogenesis in CLDs: the pro-angiogenic action of leptin and PDGF

Several peptide mediators other than VEGF, Ang-1 and HGF are likely to be involved in hepatic angiogenesis associated with the fibrogenic progression process in CLDs. Here we would like to focus attention on the "angiogenic" activity of a limited number of polypeptide mediators of ascertained relevance in progressive fibrogenesis, like leptin and PDGF.

Leptin as an "in vivo" pro-angiogenic mediator

Leptin is a circulating peptide hormone, the product of the obese (ob) gene, which is mainly produced by adipose tissue in relation to its mass (Ahima and Osei, 2004). Although originally described mainly as a satiety factor, leptin is now recognized as a pleiotropic peptide able to modulate immune function, fertility, bone formation and wound healing (Huang and Li, 2000; Faggioni et al., 2001). Pertinent to this review, leptin is also able to modulate the response to liver injury (Yang et al., 1997; Faggioni et al., 2000) and has been reported to act as a pro-fibrogenic mediator on HSC and HSC/MFs (Marra, 2002; Friedman, 2008b). The potential role of leptin as a hepatic pro-angiogenic peptide was first reported in a study showing that human HSC/MFs were able to respond to leptin (see details in section 6.2) by up-regulating both VEGF and Ang-1 expression, as well as the pro-inflammatory chemokine monocyte chemoattractant protein 1 or MCP-1 (Aleffi et al., 2005). More relevant, in the same study positive immunostaining for the leptin receptor ObR was found to colocalize with VEGF and α-SMA in fibrotic rat livers after chronic CCl_4 administration.

The profibrogenic action of leptin has been mainly involved in the pathogenesis of non-alcoholic steato-hepatitis (NASH), a very common hepatic condition in western countries, mostly found in obese and/or diabetic patients carrying metabolic syndrome, and potentially able to progress towards cirrhosis and even HCC (Angulo, 2002; Marra, 2002; Tilg and Hotamisligil, 2006; Parekh and Anania, 2007). Along these lines, in order to study the role of angiogenesis in NASH a Japanese group (Kitade et al., 2006) administered Zucker rats, animals that naturally develop leptin receptor mutations, and their lean littermates the steatogenic choline-deficient and aminoacid defined (CDAA) diet. Although both Zucker and littermate rats similarly developed a marked steatohepatitis, the most relevant message from the study was that progression to fibrosis and cirrhosis was only seen in lean littermate rats, which is the animal able to express normal leptin receptors. Lean littermate rats exposed to CDAA diet, but again not Zucker rats, were also the only animals in which progressive fibrogenesis was associated with a parallel increase in VEGF expression and hepatic neovascularisation, with CD31 neovessels mostly located along fibrotic septa.

Results from the two studies cited (Aleffi et al., 2005; Kitade et al., 2006) indeed suggest that leptin, possibly by inducing HSC/MFs to express angiogenic cytokines, may contribute to regulate neovascularization

in NASH favouring fibrosis progression.

PDGF and hepatic angiogenesis

As already outlined in section 3, PDGF has a well established pro-angiogenic role that is mainly related to the ability to recruit pericytes and mesenchymal cells to neovessels in order to favour their stabilization. In the case of liver, this is likely to involve recruitment of HSC that have been described to act as liver specific pericytes and to be extremely sensitive to both mitogenic and chemotactic action of PDGF (Pinzani and Marra, 2001; Carmeliet, 2003; Lee et al., 2007; Friedman, 2008b; Parola et al., 2008). In a very recent paper Semela and coworkes, using the PDGF-R inhibitor Imatinib and a number of elegant experimental approaches, nicely outlined that PDGF is indeed able to promote an angiogenic phenotype of HSC by stimulating a downstream signalling also involving ephrin-B2, which is fundamental in regulating the HSC-driven process of vascular tube formation "in vitro", as well as enhance "in vivo" coverage of sinusoids (Semela et al., 2008). These events are likely to significantly affect crucial pericyte-mediated vascular functions like vascular permeability and pressure regulation.

6. Involvement of HSCs and HSC/MFs in liver angiogenesis: the search for stimuli and mechanisms

In the previous paragraph a possible role for HSCs and HSC/MFs (possibly also for other hepatic myofibroblast-like cells originating from other cell sources) in modulating angiogenesis has already emerged from data and concepts provided by "in vivo" studies. In the next few sections we would like to propose that, on the basis of published literature data, HSC in physiological angiogenesis and HSC/MFs during pathological angiogenesis in CLDs represent an hypoxia-sensitive and cyto- and chemokine-modulated cellular crossroad between necro-inflammation, angiogenesis and fibrogenesis. Along these lines, since the pro-inflammatory and pro-fibrogenic role of HSC and mainly of HSC/MFs is well established (Pinzani and Marra, 2001; Bataller and Brenner, 2005; Friedman, 2008a,b; Parola et al., 2008) we will focus the analysis on HSCs as liver specific pericytes and on the dual role of HSC and HSC/MFs (as pro-angiogenic cells and as target cells for angiogenic cytokines) in relation to hepatic angiogenesis and vascular remodelling.

6.1 HSCs as liver specific pericytes

A role for HSCs as specific liver pericytes was first proposed more than fifteen years ago (Pinzani et al., 1992) and indeed there are several features and findings that support this concept and suggest a vasomotor function for these cells. First, HSCs can express phenotypic markers that are in common with other pericytes, including desmin, glial fibrillary acidic protein (GFAP), NG2 and, for activated cells, α-SMA (Geerts, 2001), and are known to respond to PDGF. Second, these cells are located in a strategic anatomical site, the space of Disse, and then in intimate contact with sinusoidal ECs by means of their characteristic perisinusoidal or sub-endothelial processes (Blomhoff and Wake, 1991; Geerts, 2001). The processes of a single HSC run along one or more adjacent sinusoids, with secondary processes being able literally to encircle the sinusoid in a cylindrical manner, making realistic the hypothesis of cells able to regulate blood flow (i.e., vasomotor cells) by modulating sinusoidal diameter. Along these lines, one should note that HSCs in normal liver are reached by axonal processes of autonomic nerve fibers that contain several vasoactive peptides (substance P, neuropeptide Y, somatostatin and calcitonin gene-related peptide). Third, several laboratories have provided evidence indicating that cultured HSC can respond to a number of vasoactive agents, including a) those able to induce contraction, like endothelin-1, angiotensin II, thrombin, vasopressin, prostaglandin F2α, thromboxane A2, substance P, platelet-activating factor (PAF) and adenosine; b) those able to induce vasodilation, like NO, carbon monoxide, prostaglandin E2 (PGE2), lipoPGE1 and adrenomedullin (Geerts, 2001; Rockey, 2001 and references therein). Even more relevant, several studies have provided evidence indicating that HSCs can contract in situ in the hepatic sinusoids (Rockey, 2001 and references therein). Moreover, there is a general agreement that in the scenario of CLDs, characterized by over-production of ET-1 (with HSC/MFs actively contributing to this event) and reduction of NO released by ECs, HSCs and HSC/MFs may contribute to the increased intrahepatic resistance and then the genesis of portal hypertension (Rockey, 2001 and references therein).

More recent data, described in the next sections, have extended this concept and suggested a role for HSC/MFs in sustaining angiogenesis and sinusoidal remodelling in CLDs (Lee et al., 2007).

6.2 HSCs and HSC/MFs as pro-angiogenic cells

The first findings suggesting that HSCs and HSC/MFs may actively contribute to angiogenesis were obtained no more than ten years ago by two different laboratories using the experimental model of CCl_4-induced acute liver injury (Ankoma-Sey et al., 1998; Ishikawa et al., 1999). These studies analysed in a time dependent way recovery from acute liver injury and, by taking advantage of morphological analysis or by detecting transcripts for angiogenesis-related molecules, both laboratories showed that VEGF expression was not limited to ECs or hepatocytes but was also detectable in mesenchymal cell types, particularly transiently activated HSCs. In one of these studies (Ankoma-Sey et

al., 1998) the authors showed that VEGF expression by rat HSCs, as isolated at different times from CCl_4 administration, was paralled by increased expression of VEGF receptors Flt-1 and Flk-1; moreover, rat HSCs in primary culture were shown to undergo increased expression of VEGF in parallel with the process of activation.

Hypoxic conditions and the angiogenic role of HSC/MFs

The latter concept (i.e., VEGF and related receptors being overexpressed by activated HSCs or HSC/MFs) was strengthened by findings coming from different laboratories that, by using either rat (Ankoma-Sey et al., 2000; Wang et al., 2004) or human (Aleffi et al., 2005; Novo et al., 2007) HSC/MFs, were all able to show that these cells, when exposed to hypoxic conditions, were able to up-regulate transcription and release of VEGF in a HIF-1α - dependent way. In T6 rat immortalized HSCs (Ankoma-Sey et al., 2000) hypoxia was able to induce all four different splice variants of VEGF described by the literature (VEGF-120, -144, -164 and -188) with up-regulation of VEGF expression being also mimicked by the release of NO from NO-donors. Where VEGF receptors are concerned, rat and human cells behaved in a slightly different way following hypoxia, with human cells up-regulating both major receptors (Flt-1 and Flk-1) and rat cells responding mainly by up-regulating VEGFR-1 (Flt-1). Exposure of human HSC/MFs to hypoxic conditions also resulted in significant up-regulation of Ang-1 and of the related receptor Tie2 (Aleffi et al., 2005; Novo et al., 2007); concerning Ang-1, it should be noted that even in normoxic conditions culture activated HSC are able to express this angiogenic cytokine (Aleffi et al., 2005; Taura et al., 2008).

Taken together, all the published data on cultured rat and human HSC/MFs can be considered as fully compatible with those reported "in vivo" (Rosmorduc et al., 1999; Corpechot et al., 2002a; Yoshiji et al., 2003; Novo et al., 2007; Taura et al., 2008; Tugues et al., 2008) indicating that indeed hypoxic conditions represent an effective stimulus for HSC/MFs to acquire a pro-angiogenic phenotype, resulting in increased transcription and release of VEGF and Ang-1.

Along these lines, one should also consider a major finding obtained some years ago, suggesting that paracrine expression of VEGF by HSCs, as well as by hepatocytes, may regulate the phenotype (i.e., fenestration and CD-31 expression) of liver sinusoidal endothelial cells (DeLeve et al., 2004).

Leptin as a pro-angiogenic mediator for HSC/MFs: facts and mechanisms.

As already discussed in section 5.4, leptin can behave "in vivo" not only as a pro-fibrogenic agent, as suggested by several studies on different animal models (Honda et al., 2002; Leclercq et al., 2002; Saxena et al., 2002), but also as a pro-angiogenic mediator, as unequivocally shown by data obtained in Zucker and lean littermate rats exposed to CDAA diet (Kitade et al., 2006) and suggested by data obtained in fibrotic livers of rats chronically treated with CCl_4 (Aleffi et al., 2005). Data obtained by analysing the action of leptin on human HSC/MFs have clearly shown how this may occur. The following concepts emerged from specifically designed experiments (Aleffi et al., 2005): a) human HSC/MFs respond to leptin by up-regulating VEGF and Ang-1 as well as the pro-inflammatory chemokine MCP-1; b) the action of leptin is possible because human HSC/MFs are able to express functional receptors (ObRs), with a detected specific increased phosphorylation for ObRb receptor isoform; c) leptin, following interaction with ObRs, stimulates several signalling pathways and transcription factors, including signal transducing and activator 3 (STAT3), NF-κB and, as also shown by others (Saxena et al., 2004), extracellular regulated kinases (ERK1/2) and c-Akt; 4) interestingly, leptin was also able to recruit and stabilize HIF-1α, leading to its nuclear translocation in a ERK1/2 and PI3-K-dependent fashion.

6.3 HSC/MFs as a profibrogenic target for the action of angiogenic mediators.

In this final section we would like to review the available literature data concerning an obvious implication of what we reported in the previous sections and paragraphs. Activated HSCs (then HSC/MFs) shows "in vivo" as well as "in vitro" the ability (for ex. responding to hypoxia and leptin) to up-regulate expression of both VEGF and Ang-1, as well as, likely, to release these mediators in the extracellular microenvironment; at the same time (Novo et al., 2007; Taura et al., 2008) these cells can also express receptors for VEGF and Ang-1 (Flk-1 and Tie-2, respectively), again following exposure to hypoxia. The question then is whether this response of HSC/MFs is solely related to angiogenesis or if it may also contribute to further sustain (in a paracrine/autocrine way) the profibrogenic attitude of HSC/MFs. In other words, are VEGF and Ang-1 able to sustain pro-fibrogenic phenotypic responses o HSC/MFs and, possibly, of all hepatic MFs? A number of experimental studies have addressed this point and here major data are summarized.

VEGF as a mitogen for HSC/MFs

The first phenotypic response analyzed by researchers in the field was the ability of angiogenic cytokine to affect proliferation of HSC/MFs. A positive answer has been documented for VEGF in activated rat HSC/MFs by different laboratories (Ankoma-Sey et al., 1998; Olaso et al., 2003; Yoshiji et al., 2003). In two of these studies it was reported that stimulation of

proliferation was significant only if cells were plated on a substrate of collagen type I, but not on other substrates resembling the normal sub-endothelial matrix of the liver (Ankoma-Sey et al., 1998; Yoshiji et al., 2003). Indeed, when human HSC/MFs were cultured just on plastic the mitogenic effect of VEGF could not be detected (Novo et al., 2007), and this may also explain another negative report for VEGF mitogenic action on rat HSC/MFs (Mashiba et al., 1999). Interestingly, it was also reported that VEGF - induced proliferation was significantly increased by concomitant treatment with bFGF (Ankoma-Sey et al., 1998). In the study by Olaso and coworkers, increased proliferation of activated HSC/MFs was found when these cells were exposed to a conditioned medium obtained from a melanoma cell line, which was found to result in up-regulation of VEGF transcription in HSC/MFs. This event was enhanced by hypoxia and blocked by using antibodies neutralizing VEGF (Olaso et al., 2003).

No other pro-angiogenic cytokine has been reported to be able to affect proliferation of HSC/MFs, as our personal experience on human HSC/MFs exposed to recombinant Ang-1 has also confirmed (unpublished results).

VEGF as a stimulus for ECM deposition but interfering with contraction.

The next step was to analyse whether angiogenic cytokines were able to affect synthesis of ECM proteins. Once again, positive results were limited to VEGF that significantly up-regulated pro-collagen type I mRNA and protein synthesis (Olaso et al., 2003; Yoshiji et al., 2003). The same conclusion can be reasonably proposed for data provided by Corpechot and coworkers (Corpechot et al., 2002a) who were able to show up-regulation of procollagen type I synthesis in rat HSC/MFs exposed to hypoxia, a procedure also resulting in VEGF up-regulation and release in the medium. However, VEGF ability to up-regulate procollagen type I was not confirmed by others when using either rat HSC/MFs (Mashiba et al., 1999) or human HSC/MFs (Novo E and Parola M, unpublished results). Moreover, once again, Ang-1 was found to be ineffective on pro-

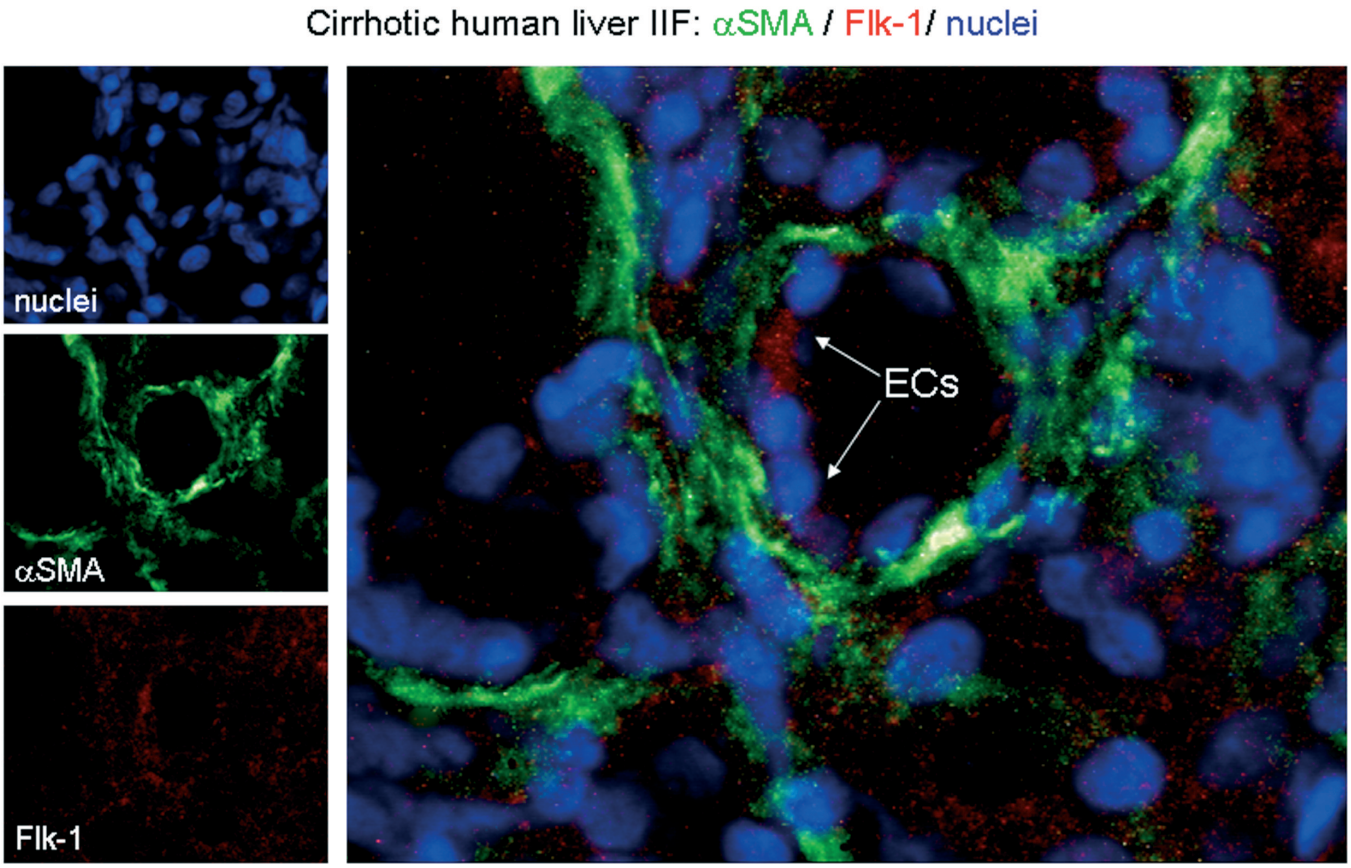

Fig. 5. In vivo localization of VEGF receptor type II or Flk-1 in a human cirrhotic liver from a HCV chronic patient. Indirect immunofluorescence on cryostat sections from cirrhotic liver of Flk-1 positive HSC/MFs (red fluorescence), of α-SMA (green fluorescence) plus DAPI nuclear counterstain (blue fluorescence).

collagen Type I synthesis.

On the other hand, a single study reported inhibition of cell contraction during "in vitro" activation of rat HSC/MFs by VEGF, an event that has been attributed to a VEGF-dependent and Flt-1-mediated attenuation of α-SMA expression (Mashiba et al., 1999).

VEGF and Ang-1 as stimuli able to induce migration of HSC/MFs

A final relevant finding that links hypoxia, angiogenic cytokines and the profibrogenic role of these cells is the observation that 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 (Novo et al., 2007), as shown by using the wound healing assay or the modified Boyden's chamber technique. The effect of VEGF on migration and chemotaxis was reported to be dose-dependent, with a chemotactic action comparable to the one exerted by PDGF-BB, used as positive control. Experimental manipulations revealed that under hypoxic conditions VEGF is progressively released by human HSC/MFs and that the action of the cytokine mainly depends on its interaction with VEGFR-2 or Flk-1 and on stimulation of Ras/Erk signalling (Novo et al., 2007) as well as on the activation of c-Jun N-terminal kinase isoforms (JNKs) (Novo et al., 2008b, 2009, submitted). Moreover, Ang-1, similarly to what was described for VEGF, was found to stimulate non-oriented migration and chemotaxis of human HSC/MFs (Novo et al., 2007). These findings indicate that in a chronic inflammatory and fibrotic environment, which is common to several CLDs of different aetiology, VEGF and Ang-1, produced and released by different cell populations in liver parenchyma, including hepatocytes and endothelial cells (VEGF) as well as pro-fibrogenic cells (VEGF, Ang-1), may then contribute to recruit HSC/MFs.

This finding is of general relevance if one considers that migration of HSC/MFs, a distinctive feature of these cells, can occur in response to a limited number of chemoattractant polypeptides or reactive oxygen species (ROS) which are generated during the development of either acute or chronic liver injury (Pinzani and Marra, 2001; Friedman, 2003, 2008a,b; Novo et al., 2006; Parola et al., 2008). However, the most relevant point is that such a peculiar feature of HSC/MFs, pro-inflammatory and pro-fibrogenic cells, which in turn are able to both release angiogenic cytokines and migrate in their presence, can offer an additional and significant explanation of why these cells may align with inflammatory and fibrotic septa (and neovessels) during fibrosclerotic progression of CLDs, representing a crucial cellular crossroad between the different biological process.

This interpretation is supported by recent "in vivo" morphological data obtained in human and rat fibrotic/cirrhotic livers, suggesting that α-SMA - positive cells (i.e., myofibroblast-like phenotype) able to

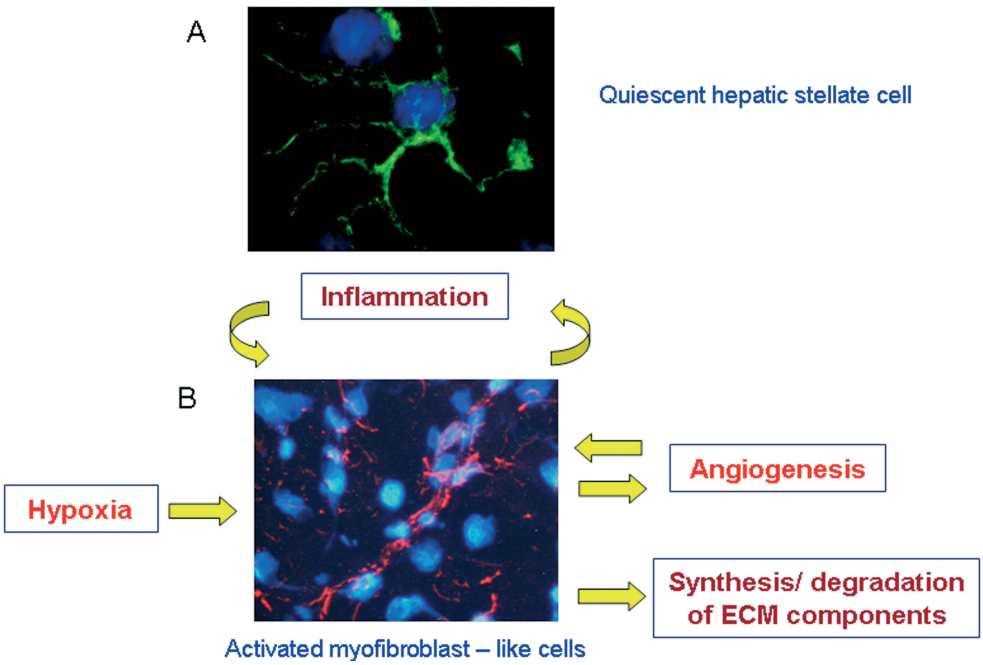

Fig. 6. Interface HSC/MFs (i.e., elements mainly found in HCV - related fibrotic/cirrhotic livers) and, possibly, other liver myofibroblasts of different origin, may represent a cellular phenotype potentially able to modulate multiple and concomitant processes, including not only fibrogenesis and inflammation but also angiogenesis and vascular/sinusoidal remodelling. From one side, these cells seem to represent a target for the multiple action of VEGF and Ang-1, including stimulation of collagen type I synthesis and recruitment of HSC/MFs and MFs, as well as, as already well established, a target for the action of pro-inflammatory mediators. At the same time, these cells are also significant sources of angiogenic cytokines, particularly under hypoxic conditions and acute and chronic liver injury, possibly through the contribution of a number of growth factors, pro-inflammatory cytokines and conditions of altered metabolic control, as recently suggested by data indicating that leptin is able to up-regulate VEGF. Morphological images. **A.** Indirect immunofluorescence of desmin positive rat HSCs on cryostat sections from normal liver (green fluorescence) plus DAPI nuclear counterstain (blue fluorescence). **B.** Indirect immunofluorescence of α-SMA positive rat HSC/MFs on cryostat sections from cirrhotic liver (red fluorescence) plus DAPI nuclear counterstain (blue fluorescence).

express concomitantly VEGF, Ang-1 or the related receptors Flk-1 and Tie-2, are found at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa (Novo et al., 2007). This distribution may indeed reflect two different phases of the angiogenic process during chronic wound healing: an early phase, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by HSC/MFs, 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 (see Figure 5), a scenario that is likely to favour the stabilization of the newly formed vessels. These findings may also suggest that the efficacy of experimental anti-angiogenic therapy (see section 5.3) in significantly preventing fibrosis progression may also rely on the block of HSC/MFs migration and chemotaxis.

7. Concluding remarks

The literature data analysed in the present review indicate that pathological angiogenesis can have a significant role in CLDs characterized by chronic injury, persisting inflammation and progressive fibrogenesis, irrespective of aetiology, with an overall scenario in which hypoxia and angiogenesis may emerge as conditions able to favour fibrogenic progression of CLDs also by sustaining the pro-fibrogenic behaviour of liver MFs. The major takehome messages from this review may be then summarized as follows:

1) Angiogenesis and fibrogenesis occur in parallel in both clinical and experimental conditions of chronic liver disease.

2) The literature data suggest that blocking angiogenesis means also blocking fibrogenesis.

3) Hypoxia should be considered as a major event in eliciting angiogenesis, with hepatocytes and profibrogenic cells being the most prominent sources of VEGF; HSC/MFs also express Ang-1.

4) HSC/MFs and, probably, activated MF-like cells of different origin, may represent (see Figure 6) a cellular crossroad of more relevant processes in chronic wound healing, both being able to release (autocrine and paracrine way) angiogenic cytokines in response to hypoxia, as well as responding to pro-fibrogenic action of VEGF and Ang-1.

Acknowledgements. Authors acknowledge financial support from the Italian Ministero dell'Università e della Ricerca (MIUR, Rome - PRIN Project 2006067527), the Regione Piemonte (Torino), the Fondazione CRT (Torino) and the Fondazione Bossolasco (Torino).

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Accepted April 13, 2009