Gastroenterol Hepatol. 2012;**35(Espec Congr 1)**:108-112

Gastroenterología y Hepatología

www.elsevier.es/gastroenterologia

XXXVII CONGRESO ANUAL DE LA ASOCIACIÓN ESPAÑOLA PARA EL ESTUDIO DEL HÍGADO

Angiogenesis in hepatocellular carcinoma development and progression

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Introduction

The discovery of the existance of blood circulation and capillaries by William Harvey and Marcello Malpighi in the 17th century is recognized as the foundation for modern medicine (Aird, *J Thromb Haemost*, 2011). Since then, the technical and scientific revolution led to an enormous gain in knowledge of the hepatic microcirculation and endothelial function in health and disease. This knowledge translated into clinical practice and helps to improve diagnostic methods and therapy of patients with chronic liver disease and hepatocellular carcinoma (HCC).

The pathogenesis of HCC is a complex multistep process leading to inactivation of tumor suppressor genes and activation of oncogenes. Molecular classifications by microarray analysis identified several HCC subtypes. However, a common feature of all HCCs is the dependence on the formation of new blood vessels to grow beyond the size of 1-2 mm^3, a process termed angiogenesis. Angiogenesis, which supplies the tumor tissue with oxygen and nutrients, is therefore a critical step in hepatocarcinogenesis, progression and metastasis of HCC. Dr. Judah Folkman (1933-2008), a pioneer in the field of tumor angiogenesis has first postulated this concept in a visionary paper in 1971. Since then, intense research has deciphered cellular mechanisms of vessel formation, molecular pathways, angiogenic growth factors and their receptors. These will be reviewed with relevance to HCC.

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Modes of tumor vascularization

Several distinct forms of angiogenesis have been discovered to contribute to the formation of tumor vasculature (Fig. 1) (Carmeliet and Jain, *Nature*, 2011). The most important

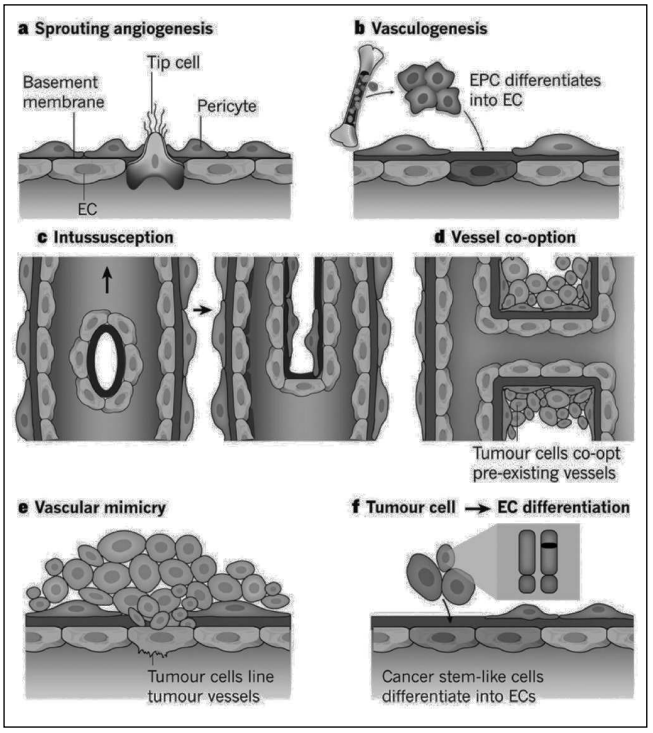

Figure 1 Modes of vessel formation.

and best-studied mode of angiogenesis is sprouting angiogenesis. Besides sprouting, tumors have evolved several other strategies to acquire neovasculature:

- *Sprouting angiogenesis*. Growth of new capillary vessels from preexisting vasculature after activation of local endothelial cells by growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and placetal growth factor (PlGF). Activated endothelial cells proliferate, migrate, form sprouts which will be recanalized and connect to the adjacent microvasculature. This primary mode of angiogenesis is found in physiological angiogenesis (i.e. liver regeneration) and pathological conditions such as tumor angiogenesis in HCC.
- *Intussusceptive angiogenesis*. Alternative mode of angiogenesis, which can contribute to the angiogenic process in addition to the classical form of sprouting angiogenesis. In contrast to sprouting, angiogenesis by intussusception, consists of microvascular remodelling by transcapillary pillar formation. Growth of these endothelial pillars leads to sinusoidal multiplication by successive fusion and partitioning of the existing vascular lumen. The split sinusoids increase in girth and undergo augmentation concomitant with endothelial cell proliferation. Intussusceptive angiogenesis has been described during liver regeneration and tumor angiogenesis in HCC (Semela, *J Hepatol*. 2007; Dill, *Gastroenterology,* in press).
- *Vasculogenic mimicry and differentiation of tumor cells to endothelial-like cells*. Dedifferentiation of tumor cells to an endothelial phenotype with formation of vascular tube-like structures ("masquerade" of tumor cells as endothelial cells). These tumor vessels are therefore not or only partially lined by endothelial cells. Vasculogenic mimicry was first described in melanoma and recently found in HCC, where it is associated with high tumor grade, invasion, metastasis and poor prognosis.
- *Vessel co-option*. Growth of tumor cells along existing vessels without evoking an angiogenic response. Vessel co-option was found in glioblastoma, lung cancer, ovarian cancer, Kaposi sarcoma and melanoma. This mechanism might be at play in early HCC since liver parenchyma with its sinusoidal microcirculation represents a highly vascularized environment for HCC.
- *Vascular remodelling*. Structural and functional modulation of the vasculature (i.e. loss of endothelial fenestrations, coverage with pericytes) as adaptive process to physiologic or pathologic stimuli. Vascular remodelling was found to be a mechanism of resistance to antiangiogenic treatment of tumor vasculature in HCC (Semela, *J Hepatol,* 2007).

Cellular regulation of angiogenesis

Endothelial cells derived from the local microvasculature are the key cells in tumor angiogenesis. However, several other cell types have been found to contribute in the process of vessels formation, stabilization and maturation (Fig. 2).

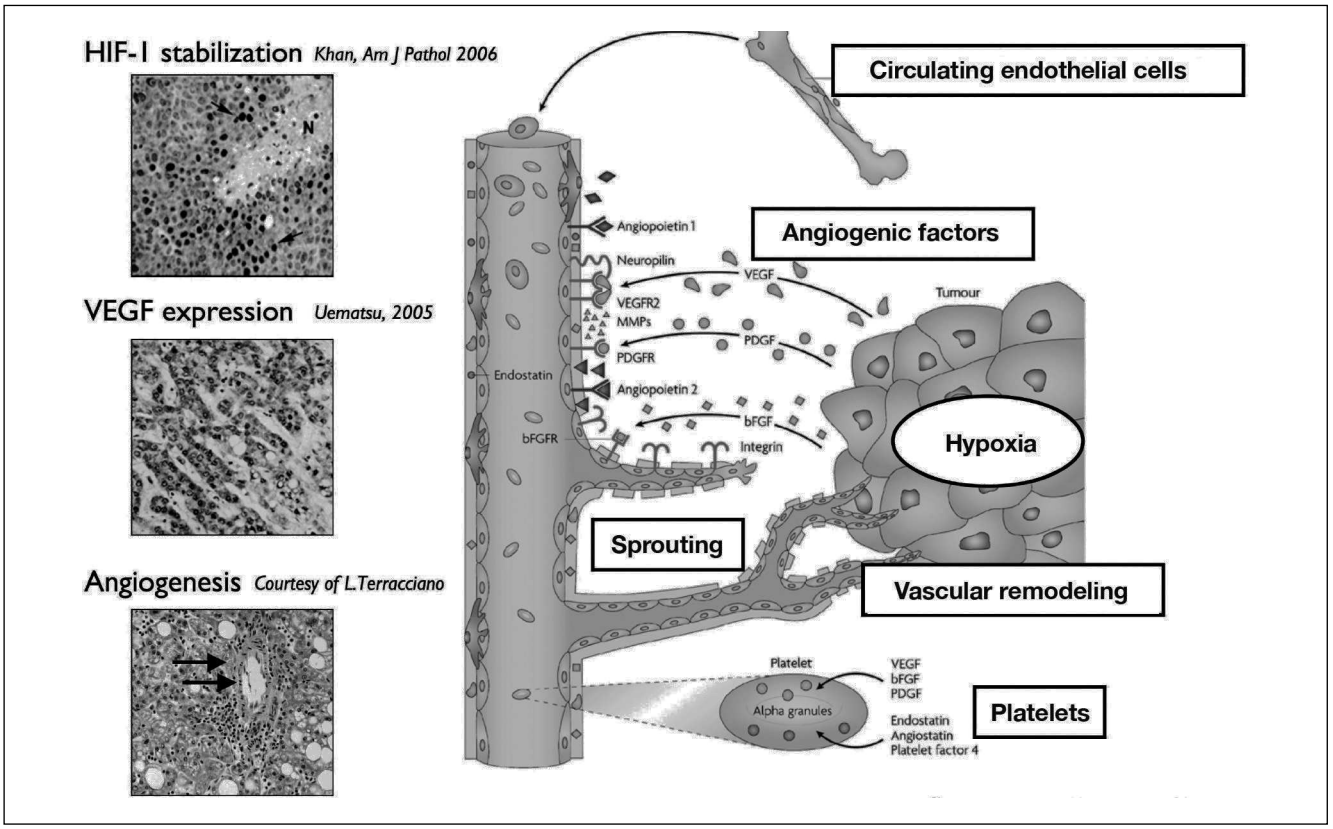

Figure 2 Key steps in tumor angiogenesis. Modified from Folkman J. Nat Rev Drug Discover. 2007.

Liver sinusoidal endothelial cells

The origin of the majority of HCC tumor endothelial cells in the liver is the compartment of the liver sinusoidal endothelial cells, which are unique microvascular cells lining the liver sinusoids characterized by fenestrations, a discontinuous endothelium and lack of an organized basement membrane. Normal liver sinusoidal endothelial cells are quiescent, with only 0.01 to 0.001% undergoing mitosis at any given time. Endothelial cell proliferation in tumor vasculature is activated leading to a turnover time of approximately 5 days. In addition, also the function and phenotype of the endothelium in HCC differs from normal liver sinusoidal endothelial cells: the endothelial cells become defenestrated, start to deposit a basement membrane ("arterialization") and upregulate the expression of different cell surface molecules (i.e. VEGFR2, CD31, CD34, CD105) which are often used to identify and quantify the tumor microvessel density by immunohistochemistry.

Circulating endothelial progenitor cells

Not all endothelial cells in tumor vessels originate from neighbouring vessels. Bone marrow derived circulating endothelial progenitor cells (EPCs, characterized by stem cell markers such as CD117 or CD133, as well as CD31, CD34 and VEGFR2) have been shown contribute to a small extent to the local tumor angiogenesis in HCC. Thereby, circulating endothelial cells are mobilized from bone marrow by systemically circulating growth factors (i.e. VEGF, PDGF) and chemokines (i.e. interleukin-8). These cells are able of homing to sites of neovascularization in the liver and HCC, integrate into the local vascular network and differentiate into mature endothelial cells. Levels of endothelial progenitor cells have been shown to be increased in patients with HCC and probably correlate with advanced tumor stage (Yu, *Clin Cancer Res,* 2007; Ho, *Hepatology,* 2006).

Hepatic stellate cells

Hepatic stellate cells are an integral component of sinusoids and function as liver-specific pericytes, which are cells that maintain and support endothelial cells. Pericytes regulate vessel stabilization, maturation and remodeling by direct contact and paracrine interaction with endothelial cells (i.e. PDGF, VEGF, nitric oxide). Pericytes have been intensively studied in tumor vasculature of different solid tumors; targeting both pericytes and endothelial cells in vasculature of experimental tumors such as HCC with kinase inhibitors showed increased efficacy in HCC growth inhibition.

Thrombocytes

Thrombocytes, besides their role in hemostasis, are important in the regulation of endothelial cells. Thrombocytes contain high concentrations of angiogenesis stimulators (i.e. PDGF, VEGF, bFGF, HGF, angiopoietin-1) and inhibitors (i.e. endostatin, thrombospondin-1, platelet factor-4 (PF-4)) packaged in distinct populations of alpha-granules which can be released selectively (Italiano, *Blood,* 2008). Further, thrombocytes are able to take up and concentrate angiogenic proteins such as VEGF and bFGF if a tumor is present in the organism. Serum VEGF per platelet count, as an indirect estimate of VEGF in platelets has been shown to correlate with tumor stage, presence of portal vein thrombosis and survival in HCC patients. Although unexplored, one can speculate that as thrombocytes adhere to tumor endothelium their action can enhance or inhibit local angiogenesis and thereby influence HCC growth.

Vascular changes in hepatocellular carcinoma

Tumor angiogenesis is distinct from physiological angiogenesis which occurs i.e. during liver regeneration: in contrast to physiological angiogenesis tumor angiogenesis in HCC is not self-limited in time and leads to formation of abnormal vessels characterized by widened lumina, aneurysmal dilatations, irregular blood flow, regions of stasis, high permeability, leakiness and bleeding as well as different expression of endothelial cell markers (i.e. CD 34) (reviewed by Yang and Poon, *Anat Rec,* 2008). Another important characteristic of the HCC microvasculature is an almost exclusive vascular supply by branches derived from the hepatic artery. This "arterialization" evolves gradually from dysplastic nodules to HCC and leads to the typical hypervascularized pattern of HCC during the arterial phase on imaging studies (contrast-enhanced ultrasound, CT, MRI) and later wash out in the portal venous phase. These vascular imaging features have been incorporated in the EASL and AASLD non-invasive diagnostic criteria for HCC.

Molecular pathways in tumor angiogenesis

A range of proangiogenic and antiangiogenic factors orchestrates angiogenesis. In tumors the balance is shifted towards proangiogenic factors ("angiogenic switch"), which are mainly produced by tumor cells and are upregulated by several mechanisms: by hypoxia through hypoxia-inducible factor (HIF)-1, by cytokines and by mutations in tumor suppressor genes and oncogenes like *p53, ras, raf, VHL, myc, c-fos*. Recently focal gains of *VEGFA* (i.e. *VEGFA* amplification, *VEGFA* high level gains and chromosome 6 gains) have been shown to contribute to increased VEGF expression in HCC (Chiang, *Cancer Res,* 2008).

Hypoxia is a potent stimulator of VEGF expression. Hypoxia-inducible factor (HIF)-1 is the key transcription factor in hypoxic tissues and induces the expression of several hypoxia-response genes such as *VEGF* and *VEGFR-1*. Hypoxia in the centre of growing tumors leads to intracellular stabilization by hydroxylation of hypoxia-inducible factor (HIF)-1α, a constitutively expressed protein which is rapidly degraded by the proteasome under normoxic conditions. Stabilized HIF-1α acts as key transcription factor in hypoxic tissues and induces the expression of several hypoxia-response genes, such as VEGF. VEGF upregulation in turn promotes cell survival, proliferation and migration of endothelial cells, which will lead to the formation of new blood vessels especially in the tumor periphery. Improved vascularization and perfusion will lead to further tumor growth with persistent central hypoxia.

A recent study analyzing the transcriptome of liver sinusoidal endothelial cells revealed several genes, which are overexpressed at least 10-fold or higher in liver tumor endothelial cells compared to liver sinusoidal endothelial in physiological angiogenesis during liver regeneration (Seaman, *Cancer Cell,* 2007): *Vscp, CD276, Ets variant gene 4, CD137, MiRP2, Ubiquitin D, Prion-PLP, Apelin, Placental growth factor, PTPRN, CD109, Progressive ankylosis, Collagen VIII*.

Mammalian target of rapamycin (mTOR) is a central serine-threonine kinase which regulates proliferation and cell cycle progression. mTOR-dependent signaling was found to be activated in experimetal and human HCC. Further, mTOR is important in proliferating endothelial cells and promotes tumor angiogenesis and HCC growth (Villanueva, *Gastroenterology,* 2008).

The extracellular matrix (ECM) is another important regulator of angiogenesis and constitues an extracellular microenvironment sensor for endothelial cells. This is especially important since the vast majority of HCC occur in a cirrhotic liver rich in ECM. ECM sequesters angiogenic factors (i.e. VEGF, bFGF). Components of the ECM are potent angiogenesis blockers and might regulate angiogenesis in chronic liver disease and in HCC: degradation of type IV and XVIII collagen by matrix metalloproteinases, elastase and cathepsins releases highly potent antiangiogenic fragments such as arrestin, canstatin, tumstatin and endostatin.

The function and regulation of the 27 endogenous antiangiogenic factors know to date, although in some cases highly expressed by hepatocytes in adults (i.e. pigment epithelium-derived factor PEDF) are poorly understood. Serum PEDF levels have been shown to be downregulated gradually in patients with chronic liver disease, cirrhosis and HCC. Detailed descriptions of molecular regulation of tumor angiogenesis can be found in the selected reviews cited below.

Angiogenic growth factors as predicitve markers in patients with HCC

Proangiogenic growth factors (i.e. VEGF, bFGF) are increased in blood of patients with HCC, whereas antiangiogenic factors (i.e. PEDF) are decreased. Circulating proangiogenic growth factors and HCC microvessel density have been shown to be predictors of HCC recurrence and survival after curative resection: Circulating concentration of VEGF increases with the stage of HCC, the highest levels being in patients with metastasis. A prospective study of 100 patients suffering from HCC found that high serum levels of VEGF significantly correlated with absence of tumor capsule, presence of intrahepatic metastasis, presence of microscopic venous invasion, advanced stage and postoperative recurrence (Poon, *Ann Surg,* 2001). In a recent study, preoperative serum VEGF in 98 patients with resectable HCC was a significant and independent predictor of tumor recurrence, disease-free survival and overall survival (Chao, *Ann Surg Oncol,* 2003). In 80 patients with inoperable HCC undergoing transarterial chemoembolization (TACE), Poon and coworkers evaluated the prognostic significance of serum VEGF levels prospectively (Poon, *Oncol Rep,* 2004): pretreatment serum VEGF levels were significantly higher in patients with progressive disease than those with stable or responsive disease. Patients with serum VEGF >240 pg/ml had significantly worse survival than those with serum VEGF <240 pg/ml (median survival 6.8 vs. 19.2 months, p=0.007). In a Cox multivariate analysis, serum VEGF >240 pg/ml was an independent prognostic factor of survival (Poon, *Oncol Rep,* 2004).

Clinical translation of antiangiogenic drugs

Years of intense vascular biology research led to the FDA approval of the angiogenesis inhibitor drugs (Table 1). In the

Table 1 FDA approved antiangiogenics 2012 and indications

Tyrosine kinase inhibitors	
Sorafenib (Nexavar)	Advanced hepatocellular carcinoma
Pazopanib (Votrient)	Advanced renal cell carcinoma
Sunitinib (Sutent)	Advanced renal cell carcinoma, gastrointestinal stromal tumors (GIST) after disease progression on, or intolerance to imatinib (Gleevec)
Antibodies targetting VEGF signaling	
Bevacizumab (Avastin)	Metastatic colorectal carcinoma, advanced NSCLC, advanced renal cell carcinoma, glioblastoma (2nd line), metastatic HER2-neg breast cancer
Ranibizumab (Lucentis)	Neovascular (wet) age-related macular degeneration
Pegaptanib (Macugen)	Macular edema after retinal vein occlusion
mTOR inhibitors	
Temsirolimus (Torisel)	Advanced renal cell carcinoma resistent to sorafenib or sunitinib
Everolimus (Afinitor)	Advanced renal cell carcinoma resistent to sorafenib or sunitinib, subependymal giant cell astrocytoma associated with tuberous sclerosis
Thalidomide	
Thalidomide (Thalomid)	With dexamethasone for treatment of multiple myeloma

Table 2 Antiangiogenic tyrosine kinase inhibitors in clinical development

Drug	Target
Sorafenib	VEGFR1-3, PDGFR, c-Kit, FLT-3, Raf/MEK/ERK
Sunitinib	VEGFR1-2, PDGFR, c-Kit, FLT-3
Pazopanib	VEGFR1-3, PDGFR, c-Kit
Axitinib	VEGFR1-3, PDGFR, c-Kit
Cediranib	VEGFR1-3
Motesanib	VEGFR1-3, PDGFR, c-Kit
Vandetanib	VEGFR2, EGFR
Indetanib (BIBF 1120)	VEGFR1-3, PDGFR, FGFR1-3
Brivanib	VEGFR2, FGFR1
XL-184	VEGFR1-3, PDGFR, c-Kit
Linifanib	VEGFR2, PDGFR, FLT-3
Dovitinib (TKI-258)	VEGFR1-3, FGFR3, PDGFR, c-Kit, FLT-3
TSU-68	VEGFR2, PDGFR, FGFR
Imatinib	PDGFR, c-Kit
Vatalanib	VEGFR1-3
RG-4733	Notch (gamma secretase inhibitor)
Foretinib	VEGFR2, c-Met, FLT-3, c-Kit
Regorafenib	VEGFR2-3, PDGFR, Ret/Kit/Raf
Plerixafor	CXCR4
PF-4217903	c-Met
JNJ-38877605	c-Met

www.clinicaltrials.gov. Modified from Teicher BA, Biochem Pharmacol 2011.

Table 3 Antiangiogenic antibodies in clinical development

Drug	Target
Bevacizumab	VEGF-A
Ramucirumab	VEGFR2
Aflibercept	VEGF-A, PIGF
AMG-386	Angiopoietin-1 & 2
Rilotumamab	Hepatocyte growth factor
AV-299	Hepatocyte growth factor
Bavituximab	Phosphatidylserine
TB-403	PIGF
REGN-421	DLL4
Anti-NRPI	Neuropilin-I
MEDI-575	PDGFR
Sonepcizumab	S-I-P
Anti-EGFL7	EGFdomain-like7
IMC-18FI	VEGFRI
TAK-701	Hepatocyte growth factor
SCH900105	Hepatocyte growth factor
MetMab	c-Met
Regorafenib	VEGFR2-3, PDGFR, Ret/Kit/Raf
Plerixafor	CXCR4
PF-4217903	c-Met
JNJ-38877605	c-Met

DLL4: delta-like ligand 4; PDGFR: platelet-derived growth factor receptor; PIGF: placenta growth factor; S-I-P: sphingosine-I-phosphate.
www.clinicaltrials.gov. Modified from Teicher BA, Biochem Pharmacol 2011.

SHARP trial, the multityrosine kinase inhibitor sorafenib (which inhibits VEGFR-1, -2, -3, PDGFR, c-Kit, FLT-3 and RAF) has been shown to prolong survival in patients with advanced HCC (Llovet, *N Engl J Med,* 2008). This study showed for the first time that systemic therapy is effective in case of advanced HCC and that patients can benefit from antiangiogenic treatment.

Several other angiogenesis inhibitors such as tyrosine kinase inhibitors (Table 2, i.e. *sunitinib, brivanib, cediranib, pazopanib, TSU-68, imatinib*), monoclonal antibodies against circulating VEGF (Table 3, i.e. *bevacizumab*), mTOR inhibitors (i.e. *rapamycin, temsirolimus, everolimus*) and vascular targeting agents (i.e. *EndoTag-1*) are currently in different phases of clinical testing for HCC.

Combination of systemic antiangiogenic drugs with other treatment modalities for HCC such as transarterial chemoembolisation (TACE), ablative therapies such as radiofrequency ablation (RFTA) and percutaneous ethanol injection (PEI) have shown promising results in animal models and are under clinical investigation in patients with HCC. However, drug toxicities, resistance to antiangiogenic drugs, markers to predict drug efficacy and optimal dosing are important issues which need to be solved (reviewed in De Bock et al, *Nat Rev Clin Oncol,* 2011; Carmeliet and Jain, *Nature,* 2011).

Suggested reading

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