Angiogenesis in human liver tumors

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CHAPTER Ⅰ

Introduction

1.1 Introducing hepatocellular carcinoma

Globally, hepatocellular carcinoma (HCC) is one of the most common malignancies. HCC mainly develops in relation with chronic liver diseases, e.g viral hepatitis B and/or C infection, or alcoholic liver disease. Chronic viral hepatitis B and C frequently progress to liver cirrhosis, which is the end stage of a diffuse scar formation process in the liver. This process has a profound impact on the hepatic pathophysiology, leading to serious multiorgan complications, including portal hypertension, hepatorenal syndrome, and portopulmonal syndrome. These multiple deteriorating effects and the fact that cirrhosis related HCC develops in a diseased liver create major limitations to the therapeutic options for an HCC.

Currently, no curative treatment is available. Surgical resection is an option in patients who have not developed serious general complications due to cirrhosis, e.g., those who are still in the early Child stages. A selected group of patients are eligible for liver transplantation. The selection is based on the so called Milan-criteria, a scoring system that allows selection of patients with the least chances for tumor recurrence. Local ablative treatment modalities, chemoembolisation or radio frequency ablation offers therapeutic options in selected group of patients depending on tumor size.[1-3]

Recent developments in molecularly targeted agents have opened new therapeutic possibilities to treat HCC. Many of these agents, that target growth factor receptors such as vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and epidermal growth factor receptor (EGFR), and consequently interfere with intracellular signaling pathways downstream of the receptor, have entered phase I/II trials. The beneficial effects of most these agents are still uncertain. Sorafenib, a multikinase inhibitor is the first agent that was reported to have significant effects on halting HCC tumor growth and increasing overall survival in Child-A cirrhotic patients.[4]

Most of these molecularly targeted agents are developed to target molecules involved in angiogenesis. Anti-angiogenic agents are thought to destroy tumor vascularization, impeding the development of tumor vessels and/or killing endothelial cells of tumor vessels. Deprived of the necessary oxygen and nutrients, arrest of tumor growth and death of tumor cells will follow. Recent evidence from clinical observations suggests that creation of a situation of stable disease is a more likely outcome of anti-angiogenic drug treatment, as only immature tumor vessels are affected by therapy. At the same time, mature tumor vessels will remain functional.[5,6] The potential efficacy of anti-angiogenic treatment in HCC depends on our knowledge on the angiogenic characteristics of this tumor.

1.2 The current concept of tumor angiogenesis

The growth of tumor beyond the size of 1-2 mm^3 is highly dependent on new blood vessels formation to keep up with oxygen and nutrients demand and for removal of metabolites. Several mechanisms for tumor vascularization have been recognized, including angiogenesis, co-option, endothelial progenitor cell recruitment, vasculogenic mimicry and mosaic vessels. These different mechanisms may exist concomitantly in the same tumor or may be selectively involved in neovessel formation in a specific tumor type.[7,8]

Angiogenesis, the formation of new vessels from pre-existing ones, is an important mechanism to explain the formation of new tumor vasculature.[9.10] Angiogenic sprouting is one of the mechanisms of neovessel formation to describe the angiogenic process. It is a complex process and includes destabilization of pre-existing vessels, proliferation and migration of endothelial cells, organization of tubular vascular structures, maturation of vessels, and remodeling of extracellular matrix (ECM). Although by far not all details of the molecular and cellular mechanisms of angiogenesis have been elucidated yet, some parts are revealed which are helpful for developing strategies for anti-angiogenesis therapy and biomarker identification to monitor efficacy of therapy.

It is now well appreciated that vascular endothelial growth factor (VEGF), angiopoietins (Ang), and their receptors are important in this process. Ang-2 is produced and rapidly released by the endothelial cells which will undergo sprouting. As a consequence, Ang-2 competes with Ang-1 which blocks the phosphorylation of the Angiopoietin receptor Tie-2. Loss of Ang-1 driven Tie-2 phosphorylation leads to loss of stability of the mature vasculature, and sensitizes the endothelial cells to growth factors. The destabilized vessels next progress to the first stage of angiogenic sprouting when VEGF is present.[11,12]

The growing endothelial cells sprout is guided by a gradient of VEGF-A, especially the isoforms bound to heparan sulphate, while the shorter isoform can support the proliferation of endothelial cells. The endothelial cells migration during angiogenesis involves chemotactic, haptotactic (the directional migration toward a gradient of immobilized ligands), and mechanotactic (the directional migration generated by mechanical forces) stimuli, the degradation of ECM, and cell cytoskeletal remodeling.[13] Release of PDGF-B by the endothelial cells forming new vessels enables recruitment of PDGF-B receptor expressing pericytes, which leads to endothelial cells survival and maturation of the new vessels.[14]

Another variant of angiogenesis exists, which is called intussusceptive microvascular growth (IMG), in which the insertion of tissue pillars into a vessel divides a vessel in two. In this process, proliferation of endothelial cells is not necessary.[15] IMG may have roles in additional vascular growth and development of complex vascular beds after the initial stage of immature

capillary network formation by sprouting.[8,16] In human primary melanoma, a high IMG, indicated by the number of connections of intraluminal tissue folds with the opposite vascular wall, is correlated to a high tumor thickness.[17]

1.3 The concept of vessel co-option

In 1987 Thompson proposed that tumors acquire their vasculature by incorporation of host tissue capillaries.[18] The current concept describes that there are two models of tumor angiogenesis, one of an avascular tumor initiation and the other of tumor initiation involving host vessel co-option.[11] First evidence for the latter mode of tumor vascularization was provided by Holash and colleagues, who showed that in an orthotopic rat C6 glioma model, the tumor cells exploited the host brain vessels in the initial phases of tumorigenesis.[19]

There are also morphological evidences both in experimental models and in human tumors suggesting that co-option of pre-existing blood vessels might persist during the entire period of primary or metastasic tumor growth. In 1997, Pezzella and colleagues studied a series of 500 primary stage I non-small-cell lung carcinomas, and found in tumors of “alveolar” growth type a preserved architecture of alveolar septa while the tumor grew in a solid fashion filling the alveolar spaces without the formation of tumor-associated stroma and new vessels. So the authors hypothesized that “if an appropriate vascular bed is available, a tumor can exploit it and grow without inducing neo-angiogenesis”.[20] There is also evidence of absence of angiogenesis in secondary breast cancer metastasis growth in lung.[21]

The liver is also a well-vascularized organ with a rich blood supply and abundant sinusoids. In 1993, Paku et al. studied the morphology of experimental liver metastases (anaplastic 3LL-HH tumors, Lewis Lung carcinoma tumor cell lines), and showed during growth of “sinusoidal-type” tumors that invading cancer cells advanced between the basement membrane and the endothelial lining of the sinusoids and evoked proliferation of endothelial cells.[22] In liver metastases from patients with colorectal adenocarcinoma evidence was provided of co-option in one of three growth patterns, the so-called “replacement” pattern. In this pattern, the reticulin of liver parenchyma was conserved within the metastases at the tumor-liver interface. In addition, the endothelial cells of the blood vessels near the interface in the metastases did not express CD34 and were not surrounded by mural cells, indicating their similarities with hepatic sinudoidal endothelial cells. The ratio of the proliferating tumor cell fraction and the proliferating endothelial cells fraction was 3-4 fold higher in the replacement-type metastases compared with the other metastases.[23]

Paku et al. described a mechanism for the development of the vasculature in “pushing-type” liver metastasis of experimental colorectal cancer.[24] It

includes the proliferation of smooth muscle actin-positive stellate cells and the formation of vascular lakes from the fusion of the sinusoids at the surface of the tumor, and the development of vessel-containing connective tissue columns that traverse the tumor.

1.4 Parameters used to measure angiogenesis

As anti-angiogenic therapy is a promising treatment for human solid tumors, evaluation of the status of angiogenesis in tumors can be used in selecting patients for anti-angiogenic therapy and in selecting the appropriate type of anti angiogenic therapy for the individual patient.

For basic cell biological studies aimed to unravel the molecular control of angiogenesis, a number of experimental assays are available. For example, endothelial cells can spontaneously form tubes when cultured in gelatin in vitro, and many in vitro assays based on this behavior have been designed after the first observation.[25,26] In these in vitro assays and in some in vivo assays, such as the corneal micropocket, direct observations of angiogenesis processes are optional, e.g. by digital photograph and quantification of capillary sprout length. These assays are crucial tools for the research on therapeutic agents that can inhibit or promote angiogenesis which can in the future be used in clinical settings.

However, in tumor tissues from patients it is impossible to observe the angiogenesis associated cellular and molecular processes directly, as biopsies represent a static moment at an unknown time point after tumor growth induction and angiogenic switching. Yet a number of features can be assessed in these biopsies in daily histopathological practice, which indirectly can provide clues on the vascular status of a tumor. For example, one can quantitate intratumoral microvessels, measure structurally aberrant tumor vessels, and assess the production of pro-angiogenic and anti-angiogenic factors. So far no validated biomarkers of angiogenesis or efficacy of antiangiogenesis therapy are available for routine clinical use.[27]

1.4.1 Quantification of intratumoral microvessels

Parameters to quantify vessels in cancer include microvascular density (MVD), total microvessel area (TVA, including Chalkley counts), and vascular branching counts.[28,29] MVD in histological sections of human tumors was for a long period of time regarded as a representation of the status of angiogenesis. However, despite its wide application in many studies, it was soon recognized that although MVD counts can assess the presence of blood vessels, it does not give an indication of the degree of angiogenesis, as the dynamics of vessel formation are not determined. Still, assessment of the MVD is useful as a prognostic factor in some human cancers,[30,31] although it should be realized that it is not per se a good measurement of the efficacy of anti-angiogenic

therapies.[32] As pointed out by Folkman and colleagues several years ago, MVD largely reflects the metabolic burden of the supported tumor cells.[33]

Two categories of endothelial cells-specific markers are used to highlight intratumoral vessels for quantitative MVD analysis. The pan-endothelial cells markers such as CD31, CD34, and vWF can be visualized in formalin-fixed tissue. Because of its robustness and ease of use, CD34 is now considered the optimal marker.[28] Other markers which indicate vascular differentiation and activation, and which can be visualized in frozen tissues, include CD105 (endoglin), Tie2, integrin αvβ3 and the complex of VEGF and VEGFR-2.

Quantification of vessels can be done by microscopy, manually or by computer assisted systems. Generally, vessels in 3 “hot spots” (defining the most vascularized areas of the tumor) are measured. The rationale of counting microvessels in “hot spots” is that these areas originated from tumor cell clones with the highest angiogenic potential and consequently, with the easiest access to the blood stream and with an increased probability of metastasis.[34] Not all researchers use “hot spots”, some measure in random areas and some use computerized scanning of the tumor as a whole in one section.[35-37] Of all these methods major drawbacks are that they are difficult to standardize, that tumor heterogeneity is not easily taken into account, and that they are prone to reader-dependent variability.

The Chalkley point overlap technique has been used as an estimation of relative microvascular areas, in which a 25-point Chalkley eyepiece graticule is applied to each hotspot. The graticule is oriented to allow it to hit the maximum number of points of interest. The Chalkley count is taken as the mean value of three hotspots. By this technique, observers do not need to make decisions whether two immunostained and adjacent structures were the reflection of one single or two separate blood vessels.[34] Chalkley counts have been suggested as the primary method for immunohistochemical evaluation of angiogenesis, because of better reproducibility and correlation with prognosis.[38-40]

1.4.2 Assessment of structurally aberrant tumor vessels

The structure of tumor vessels differs from normal vessels. Size- and shape-related parameters, microvascular shape and complexity are used to measure the abnormality of vessels.[29]

Mature vessels are covered by pericytes and have a basement membrane lining. The percentage of endothelial cells with a pericyte or basement membrane coverage can hence be used as a surrogate of vessel maturity.[28] Numerous studies use double immunohistochemical staining to colocalize endothelial cells and pericytes with an antibody for endothelial cells and another one for pericytes. Endothelial cells markers have been discussed above, while pericyte markers generally used include α-smooth muscle actin and desmin.[41,42]

Through confocal laser scanning microscopy on immuno-fluorescence

slides or normal light microscopy on immunohistochemistry slides of paraffin-embedded tissues, 3-dimensional reconstruction techniques can reveal an extensive and complex network of microvasculature of tumor and offer a useful tool to examine the spatial relationship between different components in the tumor tissues.[43,44] But the time-consuming nature together with the need for specialized equipment hampers its widespread application in daily histopathological practice.

1.4.3 Quantification of endothelial cells proliferation and apoptosis

Compared to MVD, changes in endothelial cells apoptosis and proliferation status may more accurately reflect whether tumor-associated blood vessels regression and remodeling is taking place. Double-label immunohistochemical staining with two antibodies, one for identifying endothelial cells and the other for indicating proliferation (such as anti-ki-67, anti-PCNA) is applicable on paraffin-embedded human cancer tissue, and can show the proliferative fraction of tumor cells and endothelial cells simultaneously.[45] And also on paraffin sections double-label staining with antibodies to detect endothelial cells and apoptosis (anti-active caspase 3) reveals apoptosis of endothelial cells and tumor cells simultaneously.[34]

1.4.4 Pro-angiogenic and anti-angiogenic factors, proteolytic enzymes

Other parameters used in the research field of tumor angiogenesis are quantification of angiogenic growth factors, anti-angiogenic factors and proteolytic enzymes, using various technologies including immunohistochemistry, western blot, ELISA, and quantitative or semi-quantitative RT-PCR. Zymography can be used to measure the matrix metalloproteinases (MMPs) activation. The related factors usually detected include VEGF and receptors, angiopoietins, fibroblast growth factors (FGFs), MMPs, urokinase-type plasminogen activator (uPA).

Measuring circulating or urinary levels of angiogenic growth factors is another approach thought to reflect the angiogenic status of tumor.[5,46] The pre-treatment circulating levels of VEGF, bFGF and PDGF furthermore predicted patient survival in a number of studies.[47,48] They are easy to standardize and reproduce, but release of many growth factors from platelets and other cells may hamper the clinical relevance of these measurements.[49,50] In recurrent glioblastoma patients treated daily with AZD2171 (a pan-VEGF receptor tyrosine kinase inhibitor), plasma bFGF and stromal cell-derived factor 1α (SDF1α) levels increased with the tumor escaping treatment concomitantly.[51] Soluble VEGF receptors such as VEGFR1, VEGFR2 and VEGFR3 are currently being investigated in a variety of cancer indications. More work is still needed to ascertain whether these biomarkers can predict patient survival or response to anti-angiogenic therapies.[52,53]

1.5 Angiogenesis in various tumors

Several methods are nowadays available for the histopathologist to establish the vascular dynamics of a tumor on the tissue level. Yet, one should realize that tumor angiogenesis is heterogeneous in phenotype and that the concomitant microvascular heterogeneity exists at different levels, i.e., among different tumor types, in different developmental stages of tumor growth, and even within the same tumor or within the same tumor vessel segment.[54] The mode of angiogenesis might also be influenced by the specific vascular characteristics of the organs in which the tumor develops.

Due to the lack of a clear definition, uniform criteria, and methods to assess the angiogenic status of a tumor, it is hard to compare the angiogenic activity of different types of tumors and define the precise angiogenic status of a tumor. Still, with the above described toolbox scattered studies have tried to give more insight in the matter. For example, a study in six types of different human tumors (glioblastomas, renal cell carcinoma, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas) showed high MVD phenotype in the different tumors, with glioblastomas and renal cell carcinoma having the highest average MVD. Proliferating capillary index (the ratio of the number of microvessels with proliferating endothelial cells and the total number of microvessels) was significantly different between the tumors, with glioblastomas 9.6±6.1%, renal cell carcinoma 9.4±5.2%, colon carcinomas 7.8±5.2%, mammary carcinomas 5.0±4.8%, lung carcinomas 2.6±2.5% and prostate carcinomas 2.0±1.4%. Microvessel pericyte coverage index also varies, with glioblastomas 12.7±7.9%, renal cell carcinoma 17.9±7.8%, colon carcinomas 65.4±10.5%, mammary carcinomas 67.3±14.2%, lung carcinomas 40.8±14.5%, and prostate carcinomas 29.6±9.5%.[55] From this study, we can see that each human tumor type presents itself with its own set of features of vascularization status, and glioblastomas and renal cell carcinoma had higher MVD and proliferating capillary index, and lower microvessel pericyte coverage index compared to other 4 tumors.

1.6 The aims of the studies described in this thesis

Angiogenesis is considered the most important mode of tumor growth associated neovascularization to support tumor growth and hence an interesting target for therapy. Yet, for HCC much remains to be elucidated regarding the angiogenic status and the consequent putative suitability of the various types of anti-angiogenic treatment options. Due to the unique status of vascularization of the liver it is conceivable that angiogenesis in the liver may follow a different mode than in other organs. First, the liver has a dual blood supply provided by the portal vein and hepatic artery. Second the liver is a well vascularized environment created by the hepatic sinusoids which are lined by

hepatic sinusoidal endothelial cells. These cells differ from vascular endothelial cells in that they are fenestrated and that there is no basement membrane coverage, to ensure optimal exchange possibilities between blood and hepatocytes.

Based on these unique vascular characteristics in the liver we embarked on this thesis project to investigate several aspects of vascular changes and angiogenic features in primary hepatic tumors, both malignant and benign. It is well known that the sinusoidal endothelium in HCC and benign hepatic tumors undergo phenotypic alterations, e.g. expression of CD34 and coverage by alpha smooth muscle actin positive cells. In both tumor types there is also the presence of abnormal vessels, the so called solitary arteries in HCC and hepatic adenoma and thick walled arteries in focal nodular hyperplasia. Whether these vascular changes resulted from angiogenesis following the pathways described before is not well documented on the tissue level. Therefore we addressed the following issues:

1) The angiogenic status of HCC as defined by MVD, vascular maturation, and endothelial cell proliferation and apoptosis, to enable to detect a possible correlation between these characteristics and the pattern of HCC vascularization assessed by diagnostic imaging and prognosis (chapter 2)

2) The molecular status of angiogenesis in HCC and benign liver tumors. We investigated the balances in gene and protein expression levels of members of the VEGF and Angiopoietin/Tie-2 system that are considered to be directly responsible for controlling angiogenic sprouting of blood vessels. (chapters 3 and 4)

3) COX-2 expression and its putative relation with angiogenesis in HCC. COX-2 was demonstrated to be related with tumor angiogenesis in several types of tumors with consequent therapeutic implications regarding the application of COX-2 inhibitors. (chapter 5)

By this means we tried to gain insight in the angiogenic status of primary hepatic tumors both morphologically and on the molecular level, in relation with clinical characteristics. Apart from providing new pathogenetic insight the new knowledge can form a basis for rational application of molecularly targeted therapies.

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