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Angiogenesis in benign hepatic tumors: a role for Angiopoietin-1/Tie-2?

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Abstract

Tumor growth requires vascular support which can be provided by angiogenesis. Vascular endothelial growth factor (VEGF), its receptors and the Angiopoietin (Ang)/Tie-2 system are involved in the molecular control of angiogenic sprouting. In a previous study we observed a relatively low VEGF/Ang-Tie-2 dependent angiogenic activity in hepatocellular carcinoma. In the current study we investigated the vascular changes and angiogenic status of 21 benign hepatic tumors consisting of hepatic adenoma (HCA) and focal nodular hyperplasia (FNH). We studied gene expression and protein expression profiles of VEGF-A, its receptors VEGFR-1 and -2 as well as Ang-1, Ang-2, and their receptor Tie-2, in comparison with histologically normal liver samples. The cellular localization of the proteins was investigated by immunohistology.

We observed no changes in expression of VEGF-A, nor its receptors in HCA and FNH. Ang-1 was upregulated in FNH and HCA concurrent with increased Tie-2 in FNH. In both tumor types Ang-2 levels were similar to those in normal liver samples. Immunohistologically, hepatocytes of both tumor types showed abundance of Ang-1 protein expression whereas Tie-2 was expressed by sinusoidal endothelium. CD34 and αSMA staining showed increased expression, in a sinusoidal pattern in HCA whereas in FNH this increase was present in the vicinity of the central scar and scar-like structures.

Conclusion: The unaltered VEGF-A and Ang-2 status in FNH and HCA implied that VEGF-A/Ang-Tie2 dependent angiogenesis is not occurring in these tumors. The increased Ang-1 and Tie-2 expression, the presence of sinusoidal capillarization, activated myofibroblasts, and vascular features, which are compatible with Ang-1/Tie-2 effects, suggest a role for Ang-1/Tie-2 induced vascular remodeling of the hepatic microvasculature in FNH and HCA.
Introduction

Tumor angiogenesis refers to the process of neovessel formation to provide a tumor with the necessary vascularization to grow and to metastasize in case of malignant growth. Studies on tumor angiogenesis predominantly deal with malignant tumors although benign neoplastic counterparts can be found in every organ. Some of these benign variants might represent premalignant disease, e.g. colorectal adenomatous polyps. Benign proliferative lesions would also require new vessel formation to grow; yet, studies on angiogenesis in these tumors are scarce.

In a previous study we investigated the angiogenic characteristics of human hepatocellular carcinoma (HCC), both in cirrhotic and non-cirrhotic livers. Focusing on the Angiopoietin/Tie-2 system and VEGF-A and its receptors, we observed that human HCC is devoid of any significant Ang-2 upregulation concurrent with an increase of VEGF-A expression as a prelude to VEGF-R signaling to vascular sprouting. This finding contrasted with their pattern in the highly angiogenic human renal cell carcinoma, which is known to engage in active angiogenesis via the Ang-2/Tie-2 - VEGF/VEGFR axis (1). Based on this observation we hypothesized that the absence of VEGF-A/Ang-Tie-2 driven sprouting angiogenesis in HCC is due to the fact that this malignant growth of hepatocytes is situated in a natural habitat which is a well vascularized soil provided by the hepatic sinusoidal network. It is conceivable that vascular remodeling in this rich and possibly permissive vascular soil may be fully capable of providing the required supplementary blood supply for both malignant and benign tumor growth without the additional need for robust neovessel formation.

To further investigate this hypothesis, we studied gene and protein expression levels of VEGF-A, VEGFR-1 and 2, Ang-1, Ang-2 and their receptor Tie-2 in hepatocellular adenoma (HCA) and focal nodular hyperplasia (FNH). The cellular localization of these proteins was analyzed by immunohistology and we compared the outcome with the pattern in histologically normal liver samples. Additionally, we also studied CD34 and αSMA expression in these tumors.

Patients and methods

Patients and tissue samples

Frozen tissue samples of 9 FNH (mean age 33.1±4.7), and 12 HCA (mean age 37.5±10.5) patients who underwent partial liver resection for the tumor were included. All patients were females. One patient in the HCA group had 2 separate tumors, so the total of tumor samples was 9 FNH and 13 HCA. We also included 9 samples of livers showing normal histological features. These
samples were collected from surplus material of donor liver, hemangioma liver and a traumatic liver rupture. Adjacent, non tumorous liver tissue was also included in the study. The tumors were histologically classified according to the Bordeaux update of the classification of benign hepatic nodules (2) and their phenotype was further confirmed by immunohistology using the profiles recommended by Bioulac-Sage et al (3). All lesions represented benign hepatocellular proliferative lesions in an otherwise non-diseased liver.

**Quantitative RT-PCR for mRNA analysis**

Total RNA was isolated with the RNAeasy Mini Kit (Qiagen, Leusden, Netherlands) with subsequent DNA removal using the RNase-free DNase set (Qiagen), both according to the protocol of the manufacturer. RNA was analyzed qualitatively by gel electrophoresis and quantitatively with Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Reverse transcription and real-time PCR were performed as described previously (4). Briefly, one microgram of total cellular RNA was used for the synthesis of first-strand cDNA and 10 ng cDNA was used for each PCR reaction. Exons overlapping primers and minor groove binder (MGB) probes used for real-time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping gene GAPDH (assay ID Hs99999905_m1), VEGF (Hs00173626_m1), VEGFR-1 (Hs00176573_m1), VEGFR-2 (Hs00176676_m1), Tie2 (assay ID Hs00176096_ml), Angpt-1 (assay ID Hs00181613_ml), Angpt-2 (assay ID Hs00169867_ml). TaqMan quantitative RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Amplification was performed with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. Triplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values (Ct) were averaged. Gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the relative gene expression value. Control samples of distilled water and randomly chosen RNA isolates that were not subjected to reverse transcriptase were consistently found to be negative.

**Western blot analysis**

Of each frozen tissue block, 20 samples of 5 μm thick tissue slices were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin (Sigma), and 1μg/ml leupeptin (Roche), and 1μg/ml pepstatin (Roche)].Cell debris was removed by centrifugation at 10,000g for 15 minutes, and protein concentration was measured using pyrogallol red-molybdate
solution. Indicated amounts of lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (0.45μm, Bio-Rad laboratories; Hercules, CA). The membranes were next probed with various primary antibodies (VEGF-A 1:1000, Santa Cruz sc-152, Angpt-1 1:1000 Santa Cruz sc-6319, Angpt-2 1:2000 Santa Cruz sc-7017, Tie2 1:300 Santa Cruz sc-324), diluted in 5% no-fat milk /0.1% TBST) at 4°C overnight, followed by incubation with peroxidase-labeled secondary antibodies (1:1000) and treated with an enhanced chemiluminescent substrate for detection of HRP (Amersham Life Science, London, UK). Then the membranes were stripped with 25mM glycine/1% SDS (PH 2.0) buffer, and ß-actin (mouse anti- ß-actin, 1:3000, Abcam, ab8226) was detected as loading control. Protein expression observed as electrophoretic bands in X-ray films was quantified using image analysis software (Quantity One, Bio-Rad) to calculate the volume of bands (intensity x mm²). For each protein, the volume of each sample was divided by the volume of control (ß-actin), yielding the relative protein expression value.

**Immunohistology**

The antibodies used for immunohistology, their dilutions and sources are listed in Table 1. Staining for VEGF-A, VEGF-R1 and R2, Angiopoietin-1 and 2 and Tie-2 were all performed on frozen sections. 5 μm frozen sections, dried overnight, were fixed in acetone and incubated with primary antibodies at 4°C overnight. Endogenous peroxidase was blocked with 0.08% H₂O₂ for 30 minutes, followed by incubation with horseradish peroxidase-conjugated secondary and tertiary antibodies (1:100). AEC was applied for the staining
reaction and haematoxyline for nuclear counterstaining.

The three markers for immunophenotyping HCA and FNH, glutamine synthetase (GS), serum amyloid A protein (SAA) and liver fatty acid binding protein-1 (LFABP-1) were applied on paraffin sections as were the staining with anti CD34 and anti αSMA. In short, 4μm sections were deparaffinized, and microwave pretreatment was applied except for CD34 and αSMA. After blocking endogenous peroxidase by H_{2}O_{2}, slides were incubated with the primary antibody followed by the secondary antibody. Diaminobenzidin was applied to visualize the staining reaction and hematoxylin for counterstaining.

**Results**

The benign hepatic tumors included in this study were classified according to the guidelines of the Bordeaux update and immunohistologic profiles as recommended by Bioulac-Sage et al. (2,3). All 9 samples of FNH showed the typical fern-like pattern of GS expression whereas SAA and LFABP-1 expression was similar to the pattern observed in the adjacent non-tumorous liver. The HCA group consisted of six tumors showing the immunophenotype of inflammatory type adenoma with diffuse increase of SAA expression, two samples demonstrating lack of LFABP-1, one with diffuse increase of GS and four tumors without any specific immunophenotypic characteristics.

Because angiogenic sprouting is considered to mainly originate from the microvasculature, we investigated the phenotypical changes of the microvasculature, using CD34 as a vascular endothelial marker and αSMA as the marker of pericytes and activated hepatic stellate cells or myofibroblasts.

In histologically normal livers CD34 is only expressed by vascular endothelial cells (VEC) and a small rim of periportal hepatic sinusoidal endothelial cells (HSEC) (fig 1). In FNH increased CD34 sinusoidal expression is found, mainly around the central scar and scar like structures within the tumor parenchyma (fig 1) in a decreasing gradient pattern from the scar deeper into the parenchyma. In HCA the HSEC showed increase of CD34 expression (fig 1) in a variable, non-specific pattern, either patchy or diffuse. The expression of αSMA in histologically normal livers is limited to vascular walls (fig 1). In FNH an obvious increase is seen in the stromal tissue of the central scar and scar like structures as well as the sinusoids surrounding these structures. The pattern is similar to the gradient described for CD34 expression. In HCA variable increase of sinusoidal αSMA expression is noted (fig 1), ranging from scant in the sinusoids to a diffuse increase. The αSMA staining also emphasized the presence of haphazardly distributed single arteries.

No specific patterns were observed in CD34 and αSMA expression between the different subtypes of HCA.
Table 2. Cellular distribution of the angiogenic factors by immunohistology

<table>
<thead>
<tr>
<th>Protein</th>
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<th>VEC</th>
<th>Bile duct</th>
<th>Bile ductules</th>
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A = absent; A* = absent, but sometimes present in inflammatory type HCA; N = normal liver samples; FNH = focal nodular hyperplasia; HCA = hepatocellular adenoma; HSEC = hepatic sinusoidal endothelial cells; VEC = vascular endothelial cells.

**Figure 1:** Expression of αSMA and CD34 by immunohistology.

**VEGF-A and receptors: no up-regulation in FNH and HCA.**

In figure 2 results of the quantitative expression analyses of the VEGF system are summarized. FNH and HCA showed no significant alterations of VEGF-A expression both at the gene expression and protein levels when compared with normal liver samples. A subdivision of the HCA group into the largest subgroup of inflammatory type HCA did not reveal any significant differences in both gene expression and protein levels (not shown). A similar pattern was observed for VEGF-R2 expression. VEGF-R1 gene expression in FNH was lower than in normal liver samples (figure 2).

The cellular localization of VEGF-A and both receptors was studied by immunohistology (figure 3 and table 2). VEGF-A showed a similar cellular localization in normal liver samples, FNH and HCA. VEGF-A was expressed by HSEC and VEC while hepatocytes were negative, Bile ducts and bile ductules were positive in normal livers. Weaker expression was seen in ductular
structures of FNH and when ductules were present, also in the inflammatory subtype of HCA. VEGF-A expression in HSEC of HCA was much less intense than in FNH and normal livers. In the sinusoidal spaces of HCA VEGF-A was predominantly seen in macrophages. VEGF-A was also readily visible in the stromal cells in the central scar of FNH. VEGF-R1 and VEGF-R2 showed a similar localization in FNH, HCA and normal livers. Both receptors were absent in hepatocytes. The most obvious expression of both receptors was present in sinusoidal macrophages whereas HSEC and VEC showed a weaker expression. Stromal cells, macrophages and the biliary ductules in the central fibrous scar of FNH expressed both receptors (fig 3).
Figure 3: Localization of VEGF-A and VEGFR- and R2 expression by immunohistology. Frozen 5µm sections of tissue were immunohistologically stained as described in Materials and Methods. In HCA, FNH and normal livers VEGF-A & Receptors are expressed on hepatic sinusoidal endothelial cells whereas in FNH there is also expression in stromal cells in the central scar and on ductular structures.

Ang-1 and Tie-2 expression is increased in FNH and HCA

FNH and HCA showed up-regulation of Ang-1 as compared with normal liver samples at the gene expression level, although the extent of increase was small and not visible at the protein level (figure 4). In FNH but not in HCA this pattern was also observed for Tie-2. No significant differences in gene expression were seen for Ang-2 between the tumors and normal liver samples, except for the up-regulation of Ang-2 in the adjacent liver tissue of FNH. No differences could be substantiated for Ang-1 and Tie-2 at the protein level. Ang-2 was below detection limit in Western Blot analysis. Previously, we were able to demonstrate Ang-2 protein expression in renal cell carcinoma extract (1), indicating that the experimental protocol used per se is appropriate for the detection of this protein.

In figure 5 the cellular localization of Ang-1, Ang-2 and Tie-2 is depicted. In both tumor types and normal liver samples cytoplasmic staining of Ang-1 was readily observed in hepatocytes. Less obvious expression was observed in bile ducts and ductules. Ang-1 was absent in HSEC and VEC.

Ang-2 was present by HSEC while VEC showed a less pronounced expression. Hepatocytes were negative. Ang-2 was also present in bile ducts
Figure 4: a, Relative gene expression of Ang-1, Ang-2 and Tie-2 in normal livers (n = 9), FNH (n = 9) and their respective adjacent tissues (n = 5), and Adenoma (n= 13) and adjacent liver tissue (n=4), as determined by quantitative RT-PCR. Gene expression values represent relative values adjusted to GAPDH. b, Western blot of Ang-1 and Tie-2 (N=normal liver. T=tumor. Adj=corresponding adjacent nontumorous liver). The HCA was divided into I-HCA (inflammatory type adenoma) and the non-inflammatory HCA rest-group. c, Relative protein levels of Ang-1 and Tie-2 in normal livers (n = 9), FNH (n = 9) and their adjacent tissues (n = 5), and HCA (n= 13) and adjacent liver tissue (n=4), as detected by Western blot. Values are given as mean; error bars represent the standard error of the mean. *P <.05; **P <.01. Gray bars: normal liver; black bars: tumor; white bars: adjacent to tumor.

and ductules albeit less pronounced than in the endothelial cells. Tie-2 expression was strongly positive in HSEC and VEC in all groups but no expression was observed in hepatocytes, bile ducts and ductules.

Discussion

In this study we investigated the status of factors involved in angiogenic sprouting in benign hepatic tumors with special emphasis on the expression
Figure 5: Localization of Ang-1, Ang-2 and Tie-2 expression by immunohistology. Frozen 5μm section were immunohistologically stained as described in Materials and Methods. In normal liver, FNH and HCA, Ang-1 was expressed in hepatocytes only, whereas Ang-2 and Tie-2 was expressed on vascular endothelial cells and sinusoidal endothelial cells. Ang-2 was less conspicuously expressed on sinusoidal endothelial cells in HCA than on vascular endothelial cells. Ang-1, Ang-2 and Tie-2 were also expressed on biliary structures in FNH.

status of VEGF-A and VEGF-receptors R1 and R2, and Ang-1- and 2 and their receptor Tie-2. To determine possible alterations of the hepatic microvasculature in these tumors we also investigated CD34 and αSMA expression. In histologically normal liver samples CD34 expression is only found on VEC and HSEC at the periportal margin while it is absent in HSEC in the rest of the lobule. In HCA we observed an increase of HSEC CD34 expression in a patchy or diffuse distribution whereas the increased expression in FNH was concentrated in the central scar and its neighboring sinusoids. A similar distribution pattern of increased αSMA expression was noted. Of the studied angiogenesis related factors, expression of VEGF-A, its receptors VEGFR-1, VEGFR-2 and Ang-2 with its receptor Tie-2 were all observed in the sinusoidal compartment, whereas Ang-1 was present in the epithelial component, the hepatocytes and biliary structures. In fact, the latter were found to express all studied angiogenic factors albeit in variable degrees.

According to the current view of the control of angiogenesis, transition
from endothelial cell quiescence to activated endothelial cell is a prelude to the cascade of VEGF-A driven angiogenic sprouting. Endothelial cell quiescence is maintained by Tie-2 phosphorylation due to binding of Ang-1, whereas release of Ang-2 leads to competition with Ang-1 for Tie-2 and subsequent dephosphorylation that increases endothelial responsiveness to VEGF-A and possibly other angiogenic stimuli (5). In the present study we observed, in both FNH and HCA, that neither Ang-2 gene nor protein levels were altered compared to their levels in normal liver tissue. In addition, changes of VEGF-A were undetectable in both tumor types.

These findings imply that the VEGF-Ang/Tie2 pathway is likely not the key regulatory pathway for the tumor neovascularization in FNH and HCA. Instead, we observed an increased Ang-1/Tie-2 expression at the gene expression level in FNH and Ang-1 in HCA, while both factors are also readily visualized in both tumor types by immunohistology. Our data of increased gene expression of Ang-1 in FNH and HCA present the first confirmation of the studies by Paradis et al (6, 7). This suggests that the Ang-1/Tie2 system might play a regulatory role in the angiogenic activity in these tumors, the molecular consequences of which are by now unknown. We did find concurrent Tie-2 up-regulation in FNH whereas Ang-2 showed no significant changes, which again emphasizes the possible specific induction of Ang-1/Tie-2 signaling in FNH and HCA. The multiple effects of Ang-1/Tie-2 have been summarized in several reviews (8-10). It is well established that Ang-1 plays a major role in maintaining vascular integrity by preventing endothelial cell death, recruitment of smooth muscle cells for stabilization of newly formed vessels, promoting differentiation of mesenchymal cells to vascular smooth muscle cells (reviewed in 10). The role of Ang-1/Tie-2 in vessel remodeling and angiogenesis has also been documented in several disease conditions, e.g., cardiac allograft vasculopathy, pulmonary hypertension, pathological hepatic vascular and lymphatic remodeling (11-14). In both tumor types included in the present study, new vessel formation is observed in variable abnormal patterns. FNH is characterized by dystrophic vessels containing severe myointimal hyperplasia, a morphological feature similar to Ang-1 induced allograft vasculopathy (11). The pathological vascular architecture due to transgenic expression of Ang-1 in the liver, described in the experiments by Haninec et al. are reminiscent of the vascular changes observed in HCA (14). Another animal study showed that Ang-1 might modulate hepatic circulation resulting in abnormal vessel formation but also leading to nodular parenchymal changes similar to that seen in FNH (13).

We and others have also found aberrant, increased expression of CD34 in HSEC showing dissimilar distribution patterns in FNH and HCA (15,16). This finding denotes that apart from the pathological vascular changes in these tumors HSEC has also become capillarized. The finding of increased expression of αSMA, indicating activation of hepatic stellate cells and
myofibroblasts, not only underscores this process but probably reflects the effect of Ang-1/Tie-2 signaling on these cells. Hepatic stellate cells are currently recognized as the hepatic pericytes which are recruited to sites of vascular remodeling and new vessel formation under the influence of Ang-1 stimulation (17). A recent study reported an Ang-1 mediated angiogenic role of activated hepatic stellate cells in hepatic fibrosis (18).

It is also recognized that Ang-1/Tie-2 signaling exerts its effects in a context-dependent manner (9). This was demonstrated in a recent study with two different cancer cell types. In tumor containing immature vessels Ang-1 induced sprouting angiogenesis leading to tumor growth whereas inhibition of tumor growth was observed by Ang-1 enhanced maturation of vessels in a tumor containing highly mature vessels (19). These variable effects of Ang-1 on different types of blood vessels should be taken into consideration when dealing with hepatic vasculature. HSEC and the sinusoidal space contain unique features regarding the endothelial cells characteristics, matrix constituents, the hepatic stellate cells, all of which have been reported to be involved in cellular responses to Ang-1/Tie-2 angiogenic activity. Cell-cell and cell-matrix contacts have recently been reported to be pivotal in Ang-1/Tie-2 driven vascular remodeling (20), yet much is unknown about their exact interplay in the human liver.

In conclusion, in these benign primary hepatic tumors the studied angiogenic growth factors appeared to be, directly or indirectly, related to other effects than inducing angiogenesis. Ang-1/Tie-2 induced vascular remodeling is probably an important pathway in hepatic tumor growth. As much is still unknown about the role of Ang-1 in hepatic angiogenesis, it is also rather premature to speculate on possible therapeutical options. For the benign hepatic tumors of the present study surgical resection is still the best therapeutic choice. However, cases of multiple adenomas might require another approach which could be provided by further exploration of the effects of Ang-1 on the dynamics of the hepatic vasculature.
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