Angiogenesis in liver fibrosis
Adlia, Amirah

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THE EFFECTS OF VEGF-A ON FIBROSIS-ASSOCIATED ANGIogenesis

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SUBMITTED
THE EFFECTS OF VEGF-A ON FIBROSIS-ASSOCIATED ANGIGENESIS

ABSTRACT
Angiogenesis emerges in parallel with liver fibrogenesis. Vascular Endothelial Growth Factor A (VEGF-A) is a potent angiogenic factor but its effects in liver fibrosis are unclear. We aimed to investigate the role of VEGF-A in carbon tetrachloride (CCl₄)-exposed mice in vivo and in precision-cut liver slices (PCLS) of mouse and human livers ex vivo. The correlations of fibrosis markers with the endothelial cell marker (CD31), VEGF-A and VEGF receptor 1 and 2 (VEGFR1 and VEGFR2) were tested in CCl₄-exposed mice with low (LF) and high (HF) degree of fibrosis. The mRNA expression of the aforementioned markers was also tested in human and mouse PCLS exposed to VEGF-A. In LF and HF mice, an increased CD31 protein expression was located predominantly in the fibrotic scars and a decreased VEGF-A expression was observed in HF mice. In mouse PCLS, VEGF-A enhanced the expression of CD31, VEGFR1 and VEGFR2 significantly but had no effect on fibrosis. In human PCLS, however, an increased procollagen 1α1 and VEGFR1 expression levels were observed in slices exposed to VEGF-A. In vivo results showed a strong induction of fibrosis-associated angiogenesis, but the negative correlation between VEGF-A expression and fibrosis markers suggests that VEGF-A does not enhance fibrosis. This was confirmed by ex vivo results in mouse PCLS showing that VEGF-A increased angiogenesis but had no effect on fibrogenesis in the mouse. In human PCLS, fibrosis and angiogenesis were affected by different concentrations of VEGF-A than in mouse PCLS, reflecting species differences.
1. INTRODUCTION

Hepatic fibrogenesis is characterized by chronic inflammation and changes in extracellular matrix composition with a shift to an interstitial matrix-type-containing fibril-forming collagen. Angiogenesis has become a salient part in the study of liver fibrosis since it has been shown that angiogenesis emerges in parallel with hepatic fibrogenesis. Structural and anatomical changes in the liver fibrogenesis have been proposed as one of the factors that affect liver perfusion and compromise oxygen supply. In addition, the involvement of activated hepatic stellate cells (HSC), endothelial dysfunction and inflammatory cells was hypothesized to relate with the production of pro-angiogenic factors and the creation of a supporting environment for neovascularization.

To date, the impact of angiogenesis in liver fibrosis is still a contentious concern. In most studies, angiogenesis is conceived to promote fibrosis, based on the observations that it occurs simultaneously with fibrosis and inhibition of angiogenesis attenuates the development of liver fibrosis. Nevertheless, other studies show that angiogenesis inhibition eventually aggravates liver fibrosis and decelerates fibrosis resolution. Angiogenesis is tightly controlled by the balance of pro- and anti-angiogenic factors. Most angiogenic factors have been characterized in the pathogenesis of tumor formation and some have been investigated for their involvement in liver fibrosis, including vascular endothelial growth factor (VEGF). It is generally known that VEGF is one of the most potent pro-angiogenic factors. The VEGF family consists of five homologues i.e. VEGF-A, -B, -C, -D and placental growth factor (PIGF) that bind with different affinities to their receptors, i.e. VEGFR1, VEGFR2, and VEGFR3; of which only the first two are involved in angiogenic signal transduction. VEGF-A is an indispensable factor in the induction of angiogenesis and vasculogenesis. It enhances endothelial cell proliferation, promotes vessel sprouting and branching and increases microvessel permeability. In the liver, VEGFR1 and VEGFR2 are expressed on endothelial cells and on HSC, but their effects on fibrogenesis are unclear. VEGF-A exerts its effects through binding to VEGFR2, but the signaling transduction after VEGFR1 binding remains controversial. In endothelial cells, VEGFR1 was hypothesized to act as decoy receptor that has no direct signaling output, but rather affects VEGFR2 signaling. In HSC, however, VEGFR1 may exert other effects. An increased VEGFR1 expression was observed in activated rat HSC, while VEGFR2 expression was diminished. VEGF treatment in these activated rat HSC decreased alpha smooth muscle actin (α-SMA) expression through the stimulation on signal transduction of PI3K/Akt via VEGFR1.

Since VEGF-A is a potent angiogenic activators, an increased VEGF-A expression in the fibrotic liver is expected. Nevertheless, recent studies showed that VEGF-A is decreased in...
fibrogenesis and re-augments during fibrosis resolution. To study the role of VEGF-A in fibrosis, we analyzed the expression of fibrosis markers, endothelial cell marker (CD31) as a surrogate marker for angiogenesis, VEGF-A and VEGF-receptor expression (VEGFR1 and VEGFR2) in CCl₄-exposed mice with low and high degree of fibrosis. To investigate the direct effects of VEGF-A, we also evaluated its effect in an ex vivo model of fibrosis. Precision-cut liver slices (PCLS) of mouse and human livers were used as fibrosis model. A prolonged incubation for 48 h has been shown to increase the expression of several fibrosis markers including procollagen Iα1, in particular the onset of liver fibrosis can be studied in this model. This is the first study using human and mouse PCLS to investigate fibrosis-associated liver angiogenesis, allowing us to untangle angiogenic and fibrotic processes.

2. MATERIALS AND METHODS

2.1 In vivo experiment

The in vivo liver fibrosis experiment was done in three different groups of Balb/c mice (20-22 g; Harlan), i.e. healthy control (N=6), low (N=5) and high (N=5) degree fibrosis-induced mice with 6 weeks and 8 weeks of CCl₄ respectively. CCl₄ was given twice weekly by intra-peritoneal injections with increasing doses (week 1: 0.5 mL/kg; week 2: 0.8 mL/kg and week 3-8: 1 mL/kg of a solution of 0.25 mL CCl₄/mL olive oil). The Animal Ethical Committee of the University of Groningen has approved the experimental protocols.

2.2 PCLS experiments

Healthy livers were obtained from C57BL/6 mice (N=6) and human (N=8; consisting of multi-organ human donors remaining as surgical waste after split liver transplantation (n=5), cardiac death donors (n=2), and from a patient after partial hepatectomy because of liver metastasis of colorectal carcinoma (n=1)) as described in previous studies. The sample size was calculated with GPower 3.1 (F tests: ANOVA repeated measures, within factors; α= 0.05; power= 90%; effect size was obtained from pilot experiments, for mouse= 1.98; for human= 1.53). PCLS were prepared in ice-cold Krebs-Henseleit buffer supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck, Darmstadt, Germany), 10 mM HEPES (MP Biomedicals, Aurora, OH, US0041) and saturated with carbogen (95% O₂/5% CO₂) using the Krumdieck tissue slicer. PCLS with a diameter of 5 mm and a thickness of 250 µm were incubated individually in 1.3 mL of Williams Medium E (with t-glutamine, Invitrogen, Paisley, Scotland) supplemented with 25 mM glucose and 50 µg/mL gentamycin (Invitrogen, Paisley, Scotland) at 37 °C and under continues supply of 80% O₂/5% CO₂ in 12-well plates while gently shaken.
After 1 h of pre-incubation, the slices were transferred to fresh medium and further incubated for 24 and 48 h with or without 1, 10, and 100ng/mL VEGF-A (Recombinant Murine VEGF-A and Recombinant Human VEGF-A, Peprotech, USA).

2.3 mRNA expression
The effects of VEGF-A on fibrosis and angiogenesis were determined by mRNA expression of specific markers using Real Time RT-PCR. Liver samples from healthy and CCl$_4$-exposed mice and pooled samples of three slices were snap frozen and total RNA was isolated using a Maxwell® 16 LEV SimplyRNA Tissue Kit (Promega, Leiden, The Netherlands). The amount of isolated RNA was measured with ND-1000 spectrophotometer (Fisher Scientific, Landsmeer, The Netherlands).

Reverse transcription of RNA to cDNA was performed with 2 µg RNA using Reverse Transcription System (Promega, Leiden, The Netherlands). The reaction was performed in the Eppendorf master cycler gradient at 20 °C for 10 min, 42 °C for 30 min, 20 °C for 12 min, 99 °C for 5 min and 20 °C for 5 min. The mRNA expression of each marker was determined using SYBR Green Mastermix (GC Biotech, Alphen aan de Rijn, The Netherlands). List of primers used in this study are shown in Supporting Table 1.

2.4 Immunohistochemical staining
Immunohistochemical staining was performed on cryostat sections (4 µm) of liver tissue obtained from the in vivo experiment. The sections were dried and fixed with acetone and then rehydrated with PBS and incubated with the primary antibody, i.e. anti-CD31 rat monoclonal antibody (1:200, BD Pharmingen, San Jose, USA) for 60 min. Sections were further incubated with a secondary HRP-coupled anti-rat IgG antibody (1:100, DAKO, Belgium) and followed by a tertiary HRP-coupled anti-rabbit IgG antibody (1:100, DAKO, Belgium), each for 30 min. Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma, USA) for 20 min and nuclei were counterstained with haematoxylin (Fluka Chemie, Buchs, Switzerland). α-SMA staining was performed using M.O.M kit (Vector laboratories, Burlingame, CA) with anti-α-SMA mouse monoclonal antibody (1:600, Sigma, USA) as per manufacturer’s instructions. Staining quantification was performed on sections before counterstaining by using Aperio Imagescope (Positive Pixel Count Algorithm v9) and presented value is the ratio between the total area of CD31 or α-SMA positive pixels (red) to the total area of pixels in percentage.
2.5 Immunofluorescence staining

Immunofluorescence staining was performed on cryostat sections (4 µm) of liver tissue obtained from the in vivo experiment. The sections were dried and fixed with formaldehyde solution 4% (Klinipath, Deventer, The Netherlands) for 15 min at room temperature. Permeabilization was carried out with Triton X-100 0.2% for 15 min. Sections were subsequently incubated with the primary antibody, i.e. anti-VEGFR1 rabbit monoclonal antibody (1:50; Abcam, UK) or anti-VEGFR2 rabbit monoclonal antibody (1:200; Cell Signaling Technology, USA) for 90 min. Sections were further incubated with goat anti-rabbit Alexa 488 (1:100; Invitrogen, Paisley, Scotland) for 60 min. To reduce the auto-fluorescence background, sections were incubated with Sudan Black B 0.1 % (Sigma-Aldrich) for 20 min and washed with PBS. The nuclei of the cells were stained with DAPI (Sigma-Aldrich, USA) for 10 min and were mounted with Mowiol (Sigma) on the glass slide.

2.6 Statistical analysis

A minimum of three different mice and human livers were used for each experiment. In the slice experiments, three slices per treatment were used from each liver. The results were analyzed with One Way ANOVA (in vivo) and Repeated Measures ANOVA (ex vivo), both were followed with Bonferroni’s Multiple Comparisons using GraphPad Prism 7. Data are expressed as mean ± SEM. A p-value < 0.05 was considered significant.

3. RESULTS

3.1 VEGF-A negatively correlates with fibrogenesis in CCl4-exposed mice

In low degree fibrotic mice (LF), CCl4 exposure for 6 weeks increased procollagen 1α1 and desmin, but not α-SMA mRNA expression (Fig. 1a, b, c). The continuation of CCl4 treatment increased all three markers and resulted in a high degree of fibrosis (HF). α-SMA protein expression was significantly increased in LF mice (Fig. 1d, e). Moreover, positive α-SMA staining was clearly seen along the fibrotic septa in the HF mice (Fig. 1e).
Fig. 1 Levels of fibrosis markers in healthy and CCl₄-exposed mice with low (LF) and high (HF) degree of fibrosis. Graphs depict quantification of mRNA levels for procollagen 1α1 (a), desmin (b), and α-SMA (c) as fold over control (healthy mice). (d) Percentage of stained area was determined by quantitative analysis of α-SMA staining. (e) Photomicrographs of representative α-SMA staining (red) in healthy and fibrotic mouse livers; PV = portal vein and F = fibrotic area. *p<0.05; **p<0.01; ***p< 0.001; ****p<0.0001 Data are presented as means (± SEM). Scale bars = 200 µm.

In LF and HF mice, CD31 mRNA expression was not altered (Fig. 2a), but increased at protein level (Fig. 2b). In healthy mice, a strong CD31 staining was found along arteries and larger veins, whereas faint staining was seen along the sinusoids (Fig. 2c). The CD31 expression in LF and HF mice was most prominent in the fibrotic septa but was also present in arteries, veins and sinusoids (Fig. 2c).
**Fig. 2** Levels of the endothelial cell marker CD31 in healthy and CCl₄-exposed mice with low (LF) and high (HF) degree of fibrosis. (a) Graphs depict quantification of mRNA levels for CD31 as fold over control (healthy mice). (b) Percentage of stained area was determined by quantitative analysis of CD31 staining. (c) Photomicrographs of representative CD31 staining (red) on healthy and fibrotic mice livers; PV = portal vein, A = artery, F = fibrotic area. (d) mRNA levels of VEGF-A as fold over control (healthy mice).

*p<0.05; **p<0.01; ***p<0.001

Data are presented as means (± SEM). Scale bars = 200 µm.

In HF mice, a significant reduction in VEGF-A mRNA expression was seen (Fig. 2d). We subsequently investigated the correlation between VEGF-A mRNA expression and the expression of procollagen 1α1, α-SMA, and CD31 using the data of all experimental groups (Table 1). Although VEGF-A has angiogenic properties, its expression in the fibrotic livers did not correlate with either CD31 mRNA (Table 1) or protein expression (data not shown). In contrast, VEGF-A mRNA expression was negatively correlated with procollagen 1α1 and α-SMA mRNA expression (Table 1; Supporting Fig. S2).
Table 1. Correlation between mRNA expression levels of the different markers

<table>
<thead>
<tr>
<th></th>
<th>Procollagen 1α1</th>
<th>α-SMA</th>
<th>CD31</th>
<th>VEGF-A</th>
<th>VEGFR1</th>
<th>VEGFR2</th>
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<tr>
<td>Procollagen 1α1</td>
<td>0.856****</td>
<td>0.087</td>
<td>-0.651**</td>
<td>-0.445</td>
<td>-0.751***</td>
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<tr>
<td>α-SMA</td>
<td>0.065</td>
<td>0.735**</td>
<td>0.651**</td>
<td>0.555*</td>
<td>0.743***</td>
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</tr>
<tr>
<td>CD31</td>
<td>0.217</td>
<td>0.217</td>
<td>-0.445</td>
<td>0.061</td>
<td>0.126</td>
<td></td>
</tr>
<tr>
<td>VEGF-A</td>
<td>0.065</td>
<td>0.065</td>
<td>0.126</td>
<td>0.409</td>
<td>0.629**</td>
<td></td>
</tr>
<tr>
<td>VEGFR1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.762***</td>
</tr>
</tbody>
</table>

Correlation coefficient was calculated by using Pearson’s correlation method based on the fold induction of the gene of interest as compared to control (healthy mice); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

3.2 mRNA and protein expression of VEGFR2 and VEGFR1 in LF and HF mice

A significant decrease of VEGFR1 and VEGFR2 expression was observed in HF mice, whereas in LF mice both VEGFR1 and VEGFR2 expression remained unaltered (Fig. 3a, b). In this study, we found a strong correlation of VEGF-A and VEGFR2 expression (Table 1). Furthermore, we observed a significant negative correlation between VEGFR2 expression and the expression of fibrosis markers (Table 1, Supporting Fig. S2). At protein level, a positive staining of VEGFR1 was observed along the sinusoids in healthy mice, while VEGFR2 staining was observed on sinusoids and Kupffer cells. In LF and HF mice, VEGFR1 and VEGFR2 staining was also observed in myofibroblasts in the fibrotic area. In the healthy areas of LF and HF mouse livers, VEGFR2 expression was decreased compared to healthy mice, while the VEGFR1 expression remained unaltered (Fig. 3c).
3.3 The effects of VEGF-A in mouse PCLS

In accordance with the in vivo situation, an increased (1.8 fold) CD31 expression in parallel with fibrosis markers was observed in mouse PCLS after 48 h of incubation (Fig. 4a). In contrast, VEGFR1 and VEGFR2 were both decreased after 48 h and no significant difference was observed in VEGF-A expression (Fig. 4b). Treatment with VEGF-A had no significant effect on procollagen 1α1 or α-SMA (Fig. 4c). However, VEGF-A caused a significant increase in CD31 expression when the PCLS were treated with 10 and 100 ng/mL VEGF-A (Fig. 4c), accompanied by a significant increase in the expression of both VEGFR1 and VEGFR2 (Fig. 4d).
Fig. 4 mRNA expression levels of endothelial cell, angiogenesis and fibrosis markers in the 48 h incubation of mouse PCLS. Quantification of mRNA expression of fibrosis markers (procollagen 1α1 and α-SMA) and endothelial cell marker (CD31) (a), and angiogenesis marker VEGF-A and its receptors (VEGFR1 and VEGFR2) (b) after 24 h and 48 h incubations are presented in graphs as fold of 0h control. The effects of VEGF-A on the aforementioned genes are presented in (c and d) as fold of 48 h control. *: compared to 0 h control; #: compared to 24 h incubation
Data are presented as means (± SEM).

3.4 The effects of VEGF-A in human PCLS
An 8.8-fold (compared to the 0-hour control) increased CD31 expression was observed in 48h-incubated human PCLS, which was higher than the increase in mouse PCLS (1.8 fold) (Fig. 4a, 5a). Unlike in mouse, in human PCLS, both VEGFR1 and VEGFR2 mRNA levels were significantly increased after 48h (Fig. 5b). A small increase in procollagen 1α1 expression was observed in human PCLS exposed to VEGF-A 1 ng/mL and there was no significant change in CD31 expression of PCLS treated with VEGF-A (Fig. 5c). VEGF-stimulation increased the expression of VEGFR1 in a dose-dependent manner without any change in the expression of VEGFR2 (Fig. 5d). Although CD31 mRNA expression was not altered by VEGF-A treatment, five out of eight human PCLS showed increased CD31 and VEGFR1 expression after treatment with 10 and 100 ng/mL VEGF-A (Fig. 5e, f).
The effects of VEGF-A on fibrosis-associated angiogenesis

Fig. 5 mRNA expression levels of endothelial, angiogenesis and fibrosis markers in the 48 h incubation of human PCLS. Quantification of mRNA expression of fibrosis markers (procollagen 1α1 and α-SMA) and endothelial cell marker (CD31) (a), and angiogenesis marker VEGF-A and its receptors (VEGFR1 and VEGFR2) (b) after 24 h and 48 h incubations are presented in graphs as fold of 0h control. The effects of VEGF-A on the aforementioned genes are presented in (c and d) as fold of 48 h control. Graphs e and f show the effects of VEGF-A on CD31 and VEGFR1 mRNA expression relative to the untreated slices in the individual human PCLS, respectively.

*: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.
*: compared to 0 h control; #: compared to 24 h incubation

Data are presented as means (± SEM).
4. DISCUSSION

The results in this study indicate that the pro-angiogenic factor VEGF-A does not directly affect fibrogenesis despite the fact that HSC express VEGF receptors. Some studies in mice have shown that fibrogenesis is accompanied by an increased VEGF-A expression, paralleled by angiogenesis \(^1,3,21\). However, other studies showed that VEGF-A protein and mRNA expression are decreased in experimental fibrosis in mice \(^6,14\). In line with the former, studies that targeted the VEGF/VEGFR2 pathway using VEGFR tyrosine kinase inhibitors showed decreased fibrosis \(^11,12\). Nevertheless, a recent study showed that 12-week-CCL\(_4\) treated mice failed to recover during a 4-week recovery period when VEGFR2 signaling was inhibited \(^6\). In our study, a decrease in VEGF-A expression was observed along with an increase in fibrosis. HF mice showed significantly lower expression of VEGF-A compared to healthy mice. This decrease, however, was not observed in LF mice, indicating that VEGF-A might have a transient role in this chronic fibrosis model. When the data of all individual livers of the three treatment groups were taken together, the VEGF-A expression showed a significant negative correlation with the fibrosis markers. Although this does not portray a causal relation between these two parameters, it appeared that the severity of liver fibrosis is inversely related to VEGF-A expression.

In this study, we aimed to unravel the direct effect of VEGF-A on liver fibrogenesis using PCLS as a model by exposing the slices to VEGF-A. During incubation for 48 h in the absence of VEGF-A, the CD31 expression increased significantly in parallel with the increase in the fibrosis markers, similar to the \textit{in vivo} situation. The VEGF-A expression, however, remained unchanged and the decreased expression of VEGFR2 that we observed in the CCL\(_4\)-exposed mice, also occurred in this model, implying the applicability of this model to investigate angiogenesis in the fibrotic liver. Treatment of PCLS with VEGF-A increased the CD31 expression, but did not affect procollagen 1\(\alpha_1\) and \(\alpha\)-SMA expression. Thus, although it is known that HSC express VEGF receptor 1 and 2, VEGF-A does not seem to have a direct effect on fibrogenesis. This supports the finding by Kantari-Mimoun \textit{et al.} showing that genetic ablation of VEGF in CCL\(_4\)-exposed mice did not affect fibrogenesis although it reduced angiogenesis \(^6\).

In human PCLS, we also observed an increased CD31 expression during control incubation for 48 h. In contrast to what occurred in mouse PCLS, VEGFR1 and VEGFR2 increased significantly after 48 h incubation of human PCLS. The increased expression of these receptors is consistent with previous reports in cirrhotic patients \(^6,36\). This indicates that the intertwined processes of angiogenesis and fibrosis are not identical in mouse and man and highlight the importance of using human tissue to unravel the pathogenesis of fibrosis. In order to investigate the role of VEGF-A in the human liver, we exposed healthy human PCLS to VEGF-A. We observed in five out of eight
human PCLS an enhanced CD31 and also VEGFR1 expression when treated with 10 and 100 ng/mL VEGF-A. In the other three liver samples, in which VEGFR1 expression was not stimulated by VEGF-A, also the CD31 expression was not altered. Although the VEGFR1 and the CD31 expression were not significantly correlated (results not shown), the non-responsive VEGF-A treated slices had the highest pre-incubation VEGFR1 expression level compared to the responsive ones (Supporting Table 2). It could be speculated that, since the basal expression was already high, the stimulation with VEGF-A might not further increase the VEGFR1 and the CD31 expression.

Interestingly, we observed an increased procollagen 1α1 expression in human slices exposed to the low VEGF-A concentration. Some in vitro studies have shown a direct effect of VEGF-A on proliferation, migration and transformation of human HSC but not on the procollagen 1α1 expression. The increased VEGFR1 seen in human PCLS at higher VEGF-A concentrations may have acted as decoy receptor, thus attenuating the VEGF-A effects in this PCLS. Activated HSC have higher VEGFR1 expression compared to the quiescent ones. So, if VEGF-A activates HSC and VEGFR1 acts as decoy receptor, the effects of VEGF-A on HSC are transient. Further studies are warranted to explore the role of VEGFR1 during fibrogenesis.

The increased VEGFR2 expression as observed in mouse PCLS was not observed in human PCLS treated with VEGF-A. The correlation of the expression data in CCl4-exposed mice represented in Table 1 suggests that in the mouse, VEGFR2 has a more important role than VEGFR1. VEGFR1, however, is known to be a decoy receptor that can sequester VEGF. Some reports suggest that VEGFR1 might protect the liver from CCl4-exposed injury without affecting angiogenesis. The exact role of VEGFR1 and VEGFR2 in liver fibrosis is yet to be resolved.

In conclusion, this study shows that the potent pro-angiogenic factor VEGF-A has no direct effect on fibrogenesis. Angiogenesis is a prominent feature during liver fibrosis, but neither in vivo correlation studies in mice nor ex vivo studies in mouse and human tissue show a direct effect of VEGF-A on fibrogenesis. Our studies do show significant alterations in VEGF-A expression during liver fibrosis and changes in CD31, VEGFR1 and VEGFR2 expression induced by VEGF-A. This implies a well-regulated homeostatic response that needs further investigation. Our findings support the study of Kantari-Mimoun et al. and Yang et al. who concluded that VEGF-A involvement in fibrosis was not due to direct effects on fibrogenic cells, but on the infiltration of pro-fibrotic immune cells. Since blood cells are not present in our ex vivo model, the effect of immune cells infiltration is not included in our PCLS studies. However, by combining in vivo data in mice with ex vivo results in liver slices obtained from mice and human tissue, important insights
into the intertwined processes of angiogenesis and fibrosis and species differences therein can be obtained.

ACKNOWLEDGEMENTS

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REFERENCES


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SUPPORTING INFORMATION

METHOD
Viability

ATP level was used to assess the viability of the slices. After the incubation, the slices were transferred to a 1 mL sonication solution, containing 70% ethanol and 2 mM EDTA, and snap-frozen in liquid nitrogen and stored at -80 °C. ATP was measured in the supernatant of samples homogenized in a minibead beater for 45s and centrifuged at 4°C and 13,000 rpm for 5 min, using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany). ATP values (pmol) were normalized to the total protein content (µg) of the slice estimated by Lowry (Bio Rad, Veenendaal, The Netherlands).

TABLES

Supporting Table 1. Primers used in this study

<table>
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<tr>
<th>Species</th>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>GAPDH</td>
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Supporting Table 2. VEGFR1 mRNA expression relative to GAPDH in pre-incubated human PCLS (sorted from highest to lowest VEGFR1 mRNA expression relative value)

<table>
<thead>
<tr>
<th>Sample</th>
<th>VEGFR1 mRNA expression (relative to GAPDH)</th>
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<tr>
<td>Liver 2</td>
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<tr>
<td>Liver 5</td>
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</tr>
<tr>
<td>Liver 7</td>
<td>0.067</td>
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<td>Liver 8</td>
<td>0.065</td>
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<tr>
<td>Liver 1</td>
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<td>Liver 3</td>
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<td>0.027</td>
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<td>Liver 6</td>
<td>0.024</td>
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</table>
Supporting Fig. S1 The viability of mouse (a) and human (b) PCLS were not altered by VEGF-A treatment. The determination of viability was carried out by measuring ATP in the PCLS. Three slices per treatment were measured individually and presented as the average of pmol ATP per µg total protein in each experiment.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Data are means (SEM)

Supporting Fig. S2 Correlation between mRNA expression of VEGF-A, VEGFR-2 and fibrosis markers (procollagen 1α1 and α-SMA) in LF and HF mice