PRECISION-CUT LIVER SLICES AS AN ALTERNATIVE MODEL TO STUDY FIBROSIS-ASSOCIATED ANGIogenESIS

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MANUSCRIPT IN PREPARATION
ABSTRACT

Liver fibrosis is typically featured with angiogenesis. One of the challenges in studies on angiogenesis in liver fibrosis is the lack of suitable in vitro assays as the currently available assays were developed to study angiogenesis in tumors. Therefore, we aimed to develop a specific ex vivo assay for fibrosis-associated angiogenesis using a fibrosis model of rat precision-cut liver slices (PCLS). As a first step, to evaluate angiogenesis in the early onset of fibrosis, PCLS were incubated for 48 h and the mRNA expression levels of CD31 and procollagen 1α1 were measured. Angiogenesis was stimulated with vascular endothelial growth factor (VEGF-A) and inhibited with sunitinib to verify the model. An increased CD31 mRNA expression was observed in PCLS exposed to VEGF-A, while sunitinib decreased CD31 and procollagen 1α1 mRNA expression.

Secondly, to determine the formation of new intrahepatic blood vessels, PCLS were incubated for five days in two different media, i.e. RegeneMed® (Reg) and Williams’ Medium E (WME) and the CD31 protein expression was determined using immunofluorescence staining. An increased CD31 expression in Reg-incubated PCLS was observed after five days, while the expression in WME remained unchanged. Thirdly, a three-dimensional angiogenesis assay was developed by embedding PCLS in a collagen matrix and analyzing the outgrowth of cells from the tissue. Outgrowth of cells was observed after five days of culture in WME resulting in tubular structures, which were CD31 positive. In conclusion, we have developed a new model to evaluate liver-specific angiogenesis in an ex vivo model of fibrosis. The response of the model towards pro- and anti-angiogenic factors, the parallel increase in angiogenesis along with the fibrosis, and the evidence of microvessel sprouting indicate that this model can be used to investigate fibrosis-associated angiogenesis.
1. INTRODUCTION

Liver fibrosis is a reversible wound healing process which is characterized by the scarring of the liver due to the excessive accumulation of extracellular matrix proteins, predominantly collagen. Besides an increased collagen deposition, liver fibrosis is characterized by the formation of new blood vessels also known as angiogenesis. Hypoxia and inflammation have been postulated as major inducers of angiogenesis. In addition, the increased expression of some cytokines and growth factors during wound healing in liver fibrogenesis also affects the proliferation of endothelial cells which is a fundamental step in angiogenesis. Although angiogenesis is crucial for tissue growth and development, it is still unclear whether fibrosis-associated angiogenesis contributes to the progression of the disease or promotes tissue repair. Some studies demonstrated that inhibition of angiogenesis attenuated the development of liver fibrosis. In contrast, other studies showed that angiogenesis inhibition aggravated liver fibrosis and decelerated fibrosis resolution.

Further definition of the role of angiogenesis in liver fibrosis and vice versa would benefit from a model that can reflect both processes. Although there are a number of in vitro and in vivo assays available to study angiogenesis, these assays have several limitations that hamper their applicability in liver fibrosis-associated angiogenesis studies. The main limitation of these in vitro assays is that they do not reflect the distinctive features of hepatic angiogenesis that are substantially different from homologue processes in other organs or tissues. One of the unique features of the liver is the presence of two different microvascular structures, i.e. sinusoids lined by fenestrated endothelium versus large vessels lined by a continuous one. This is why hepatic angiogenesis cannot be simply evaluated with a tube formation assay using human umbilical vein endothelial cells (HUVEC), because HUVEC are isolated from macrovasculature. Another feature of fibrosis-associated hepatic angiogenesis is the possible role of hepatic stellate cells (HSC) and other liver myofibroblasts (MF) as the key players in liver fibrogenesis. These cells produce several growth factors that also stimulate angiogenesis, for instance vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), etc. This implies that to study the role of angiogenesis in liver fibrosis ideally, a liver model should contain the liver microvasculatures and at the same time provide a fibrotic milieu. In vivo assays provide the information of the integrated processes involved in angiogenesis. However, the interpretation of in vivo experiment is generally complex, and the experiment is relatively expensive because it often requires large number of animals and it can cause significant discomfort to the animals.

In order to overcome the drawbacks of the currently available angiogenesis assays, we explored the possibility of using precision-cut liver slices (PCLS) to investigate fibrosis-associated...
angiogenesis. PCLS contain all different liver cells that play role in the development of liver fibrosis, embedded in their natural extracellular matrix. PCLS have been well-validated as a model to study the early onset of fibrosis\textsuperscript{16-19}. It has been shown that during culturing for 48 h, PCLS spontaneously develop characteristics of fibrotic liver tissue, such as deposition of collagen and increased expression of collagen 1, alpha smooth muscle actin and HSP47 mRNA, which can be reverted by anti-fibrotic compounds\textsuperscript{16-19}. A recently published study on rat PCLS incubated for five days showed a high increase of collagen deposition with maintained viability, intact morphology, and function of the parenchymal and non-parenchymal cells, which was influenced by the type of the culture medium\textsuperscript{20}. However, the fibrosis model of PCLS has never been used to study fibrosis-associated angiogenesis. Therefore, we aimed to develop a novel liver-specific ex vivo assay as an alternative model to study fibrosis-associated angiogenesis.

As a first step, angiogenesis and fibrosis were evaluated by the gene expressions of the respective markers of both processes after 48 h culturing of the PCLS. The angiogenesis response of the model was tested by treating the PCLS with the pro-angiogenic factor VEGF-A and the anti-angiogenic compound sunitinib. VEGF-A is one of the most important factors in the induction of angiogenesis. It enhances endothelial cell proliferation, promotes vessel sprouting and branching and increases microvessel permeability\textsuperscript{21}. Sunitinib is a general multi-targeted receptor tyrosine kinase inhibitor targeting the VEGF receptor 2 (VEGFR2) and the PDGF receptor beta (PDGFR\beta)\textsuperscript{22}. Since both of these receptors are important in the stimulation of angiogenesis, sunitinib is widely used and known as angiogenesis inhibitor\textsuperscript{22}. Secondly, the protein expression of markers that indicate the presence of endothelial cells (CD31 and SE-1) and the effects of different culture media were evaluated in slices incubated for five days. Finally, slices were embedded in collagen to serve as a matrix for outgrowth of microvessels at the surface of the fibrotic slices cultured for five days. The outgrowth from the PCLS was evaluated by light microscopy and the presence of microvessel outgrowth was demonstrated with CD31 immunofluorescence staining. The effect of VEGF-A and sunitinib and the influence of the different media on the outgrowth were evaluated.

2. MATERIALS AND METHODS

2.1 Preparation of PCLS

The precision-cut liver slices were prepared according to the protocol by de Graaf et al\textsuperscript{23}. The livers were excised from adult male Wistar rats (~300 g; Harlan PBC, Zeist, The Netherlands) that were anesthetized under 2\% isofluorane/O\textsubscript{2} (Nicholas Piramal, London UK) and collected in ice-cold University of Wisconsin solution (UW/ViaSpan, 4 °C, Du Pont). The liver cores were
prepared by using a drill with a hollow bit (5 mm) and transferred to the cylindrical core holder of the Krumdieck Tissue Slicer (TSE systems, Sophisticated Life Science Research Instrumentation). Slices of 5 mg wet weight and a thickness of approximately 250 µm were cut and collected in ice-cold Krebs-Henseleit buffer (KREBS), supplemented with 25 mM NaHCO₃ (Merck), 25 mM D-glucose (Merck, Darmstadt, Germany), 10 mM HEPES (MP, Biomedicals, Aurora, OH, USA), saturated with carbogen (95% O₂ / 5% CO₂, pH 7.4).

2.2 mRNA expression of fibrosis and angiogenesis markers in PCLS model of the early onset fibrosis

Slices were individually pre-incubated for 1 h in 1.3 mL of Williams’ Medium E (WME) (with t-glutamine, Invitrogen, Paisley, Scotland) supplemented with glucose (final concentration 25 mM) and 50 µg/mL gentamycin (Invitrogen, Paisley, Scotland) in 12-well plates at 37 °C and under continues supply of 80% O₂ / 5% CO₂ while gently shaken. After pre-incubation, the slices were transferred to fresh medium and further incubated for 24 and 48 h with or without 1, 10, and 100 ng/mL recombinant rat VEGF-A (Peprotech, USA) and with or without 0.1 and 0.2 µM sunitinib (Sellekchem, Houston, USA). Medium was refreshed every 24 h.

The effects of VEGF-A and sunitinib on fibrosis and angiogenesis were determined by mRNA expression of specific markers using Real Time RT-PCR. Pooled samples of three rat liver 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 a 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 Table 1.

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Procollagen 1α1</td>
<td>AGCCTGAGCCAGCAGATTGA</td>
<td>CCAGGTTGCAGCCTTGTTA</td>
</tr>
<tr>
<td>CD31</td>
<td>GAAATGGTGCTTCGGTGCTC</td>
<td>GCTTCGGAGACTGGTCACAA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGCTGGTGCTGAGTATGTCG</td>
<td>CTGTGGTCATGAGCCCTTCC</td>
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2.3 Protein expression of endothelial cell markers in PCLS incubated for five days

After 1 hour of pre-incubation, slices were incubated individually in 1.3 mL of two different types of medium, i.e., WME (with t-glutamine, Invitrogen, Paisley, Scotland) supplemented with 25 mM glucose and 50 µg/mL gentamycin (Invitrogen, Paisley, Scotland) or RegeneMed® medium (Reg) (WME supplemented with RegeneMed® additives (L3STA), antibiotics (L3MAB) and supplements (L3TS) in ratio 100:15:1:2.5 (RegeneMed®, San Diego, CA, USA)) at 37 °C and under continuous supply of 80% O₂/5% CO₂ in 12-well plates while gently shaken. The incubation was carried out for five days with daily medium replacement. Cryosections of the samples were prepared by embedding fresh slices in KP-CryoCompound (Klinipath, Deventer, The Netherlands) followed by freezing in 2-methylbutane (Sigma-Aldrich, Germany) at -80 °C. Sections (4 µm) were obtained from cutting the slice perpendicular to its surface with a CryoStar NX70 cryostat (Thermo Fisher Scientific, Germany).

2.4 Three-dimensional ex vivo angiogenesis assay in collagen-embedded PCLS

2.4.1 Preparation of collagen matrix

The collagen matrix was prepared on ice by adding type I collagen (Gibco) to a mixture of distilled water, 1 N NaOH and 10x PBS to achieve the required concentration. It is important to homogenize the mixture well, because failure to mix adequately will cause uneven polymerization of the collagen. pH optimization was done by varying the amount of NaOH 1N added to the mixture. In this study, we tested the influence of different collagen concentrations (1, 2, and 3 mg/mL) and different pH (7.0 and 8.0) on sprout formation and matrix firmness. Matrix firmness was evaluated from the capability of the matrix to keep the slices embedded.

2.4.2 Embedding in collagen

After 1 h of pre-incubation, slices were embedded individually in collagen matrix on a circular coverslip (19 mm diameter; VWR, Istanbul, Turkey) and put on the bottom of each well of a 12-well plate. It is important that the slices are not too wet, because this can dilute the collagen and inhibit the firming of the matrix. After embedding of the slices, the plates were incubated at 37 °C with 80% O₂/5% CO₂ humidity in a non-shaking incubator for 2 h to allow polymerization. When the matrix was firm, 1.3 ml of WME or Reg medium was added to the embedded slices. To test the response of the model on pro- and anti-angiogenic compounds, the embedded-slices were treated with or without 20 ng/mL recombinant rat VEGF-A (Peprotech, USA) and/or 0.2 µM sunitinib (Sellekchem, Houston, USA). The incubation was carried out for five days and medium was refreshed every 24 h. Daily observation of the sprouts was carried out by using light microscopy.
2.5 Immunofluorescence staining

Immunofluorescence staining was performed on sections of slices that were incubated for five days and on whole mount collagen-embedded slices. Cryosections (4 µm) of five-day incubated slice were dried and fixed with 4% formaldehyde solution in PBS (Klinipath, Deventer, The Netherlands) for 15 min and then washed in PBS. Permeabilization was carried out with Triton X-100 (0.2% in PBS) for 15 min. The staining of two endothelial cell markers, CD31 and SE-1, was performed. Whole mount staining was done on intact collagen-embedded slices. Slices on the coverslips were fixed with 4% formaldehyde solution for 20 min and subsequently washed with sterile water. The slices were stored in the last washing step at 4°C for at least 24 h. Thereafter, the embedded slices were removed from the well together with the coverslip. The staining of CD31 and BS1 lectin was performed on whole mount section. For CD31 staining, permeabilization was performed with Triton X-100 (0.2% in PBS) for 15 min.

Sections and slices were subsequently incubated with the primary antibody in an appropriate dilution, i.e. anti-CD31 mouse monoclonal antibody (1:50; BD Biosciences, USA), hepatic sinusoidal endothelial cells (anti-SE-1 mouse monoclonal antibody, 1:100; IBL, Japan) and Rhodamine-conjugated Bandeiraea Simplicifolia Lectin (BS1 lectin, 0.1 mg/mL; Vector Laboratories, USA) as marker for the luminal side of endothelial cell for 90 min. For CD31 and SE-1 staining, sections were further incubated with goat- anti-mouse-Alexa 488 (1:100; Invitrogen, Paisley, Scotland) for 1 h. To reduce the autofluorescence background, sections and slices were incubated with 0.1% Sudan Black B (Sigma-Aldrich) for 20 min and washed with PBS. The nuclei of the cells were stained with DAPI (Sigma-Aldrich, USA) and both sections and slices were mounted with Mowiol (Sigma) on the glass slide.

An epifluorescence microscope (Leica AF6000) was used to obtain images from sections of five-day incubated slices and from whole mount collagen-embedded slices that were incubated for five days and a confocal microscope (Leica SF8) was used to obtain images from whole mount sections of collagen-embedded slices. The number of blood vessel in five-day incubated slices was indicated by the intensity and the area of pixels marked above a threshold of CD31 positive staining which was measured using ImageJ (Particle Analyzer) and normalized with the total area covered by DAPI.

2.6 Statistics

For each experiment, 3 slices each prepared from three to five different rat livers were used. The results were further analyzed with Repeated Measures ANOVA (two-day incubated PCLS) and One Way ANOVA (five-day incubated PCLS), both were followed with Dunnett’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 mRNA expression of fibrosis and angiogenesis markers in PCLS incubated for 48 h

A significant increased expression of procollagen 1α1 as fibrosis marker was observed after 48 h of PCLS incubation (Fig. 1A), while CD31, as a marker for the endothelial cell, decreased after 24 h and increased again after 48 h of incubation reaching the same level as the 0 h control (Fig. 1A).

Fig. 1 mRNA expression levels of angiogenesis and fibrosis markers in the 48 h incubation of rat PCLS. (A) mRNA expression levels of the CD31 and procollagen 1α1 after 24 h and 48 h incubations are presented in graphs as fold of 0 h control. The effects of VEGF-A (1, 10, and 100 ng/mL) and sunitinib (0.1 and 0.2 µM) on CD31 (B) and procollagen 1α1 (C) are presented as fold of 48 h control. Statistical analysis was done on ΔCt (with GAPDH as housekeeping gene) by Repeated Measures ANOVA and Dunnett’s Multiple Comparisons. (D) Correlation graph between CD31 and procollagen 1α1 mRNA expression in VEGF-A treated PCLS. Correlation coefficient (r) was calculated with Pearson’s correlation method based on the fold induction of the gene of interest.

*p<0.05; **p<0.01; ***p<0.001. Data are presented as means (±SEM); n=5

In order to validate this model as a tool to study fibrosis-associated angiogenesis, we tested the response of PCLS on pro- and anti-angiogenic treatments. VEGF-A and sunitinib were used to stimulate and inhibit angiogenesis respectively. Slices exposed to a high concentration of VEGF-
A had a significantly increased CD31 expression (Fig. 1B). A concentration-dependent increase was also observed in procollagen 1α1 expression, but it was not statistically significant (Fig. 1C). Yet, procollagen 1α1 expressions in the slices treated with VEGF-A showed a significant correlation with CD31 expression (r=0.811; P<0.0001; Fig. 1D). Sunitinib did not significantly affect the CD31 expression in slices that were not treated with VEGF-A, but it significantly decreased the CD31 expression in slices treated with 1, 10 and 100 ng/mL VEGF-A (Fig. 1B). Treatment with sunitinib also significantly decreased the procollagen 1α1 expression in slices treated with or without VEGF-A (Fig. 1C).

3.2 Protein expressions of endothelial cell markers in PCLS incubated for five days
In a previous study, our group published the successful maintenance of the viability of rat PCLS during five days of incubation in an enriched medium (Reg) developed to maintain differentiation in cultured hepatocytes, and in standard medium (WME) 20. After five days of incubation, development of fibrosis was observed in slices which was characterized by an increased number of myofibroblasts and increased deposition of collagen I and III 20. In the present study, immunostaining was used to measure the level of endothelial cell marker expression on these five-day-incubated rat PCLS. Two markers were tested in this study, i.e. CD31 and SE-1. In slices that were not incubated (0 h), a faint CD31 staining (green) was observed along the sinusoids (Fig. 2A). An increased CD31 staining intensity was seen in slices starting on the third day of incubation in Reg medium (Fig. 2A, B). This increased CD31 staining intensity was unaltered after five days of incubation. The CD31 expression in slices incubated in WME medium, however, remained unchanged (Fig. 2A, B).
Fig. 2 CD31 expression on five-day (120 h) incubated slices in Williams’ E (WME) and RegeneMed® (Reg) medium. (A) Images obtained from epifluorescence microscope depicting the CD31 staining (green) on 0, 48, and 120 h incubated slices. Cell nuclei were stained with DAPI (blue). (B) The intensity of green staining was quantified with ImageJ and normalized with the total area stained by DAPI, while graph (C) showed the number of blood vessels on the slices that was quantified based on the total area of CD31 positive pixels normalized with total area stained by DAPI.

Statistical analysis was done by One Way ANOVA and Dunnett’s Multiple Comparison test; *p<0.05; **p<0.01; ***p<0.001. Data are presented as means (±SEM).

Scale bar = 100 µm.

Since an increased intensity of CD31 stained structures does not necessarily indicate an increased number of blood vessels, we estimated the number of blood vessels in slices based on the area covered by the CD31 positive pixels. We observed a similar trend in this parameter as in CD31 staining intensity (Fig. 2B, C). Slices incubated in Reg showed a significantly increased number of CD31 positive counts starting on the third day of incubation, while slices incubated in WME medium did not show any notable changes (Fig. 2C).
Fig. 3 SE-1 expression on five-day (120 h) incubated slices in Williams’ E (WME) and RegeneMed® (Reg) medium. Images obtained from epifluorescence microscope depicting the SE-1 staining (green) on 0, 48, and 120 h incubated slices. Cell nuclei were stained with DAPI (blue). Scale bar = 50 µm.

Another endothelial cell marker used in this study is SE-1 which is a specific marker for rat sinusoidal endothelial cells. In slices that were not incubated (0 h), immunostaining of SE-1 was observed along the sinusoids (Fig. 3). Unlike the CD31 expression, the SE-1 expression was notably decreased after 48 h incubation in both WME and Reg medium (Fig. 3). The SE-1 expression was completely gone after 120 h of incubation and no difference was observed on the slices incubated with WME and Reg medium (Fig. 3).

3.3 Cellular outgrowth in collagen-embedded PCLS

In the present study, we developed a novel assay using PCLS embedded in collagen matrix. This method was developed based on the rat and mouse aortic ring assays that are commonly used in angiogenesis studies.26 We first optimized the assay by trying different collagen concentrations and varying the pH of the mixture to generate an optimum collagen polymerization, while slices remained embedded during the five days of incubation. After three days of incubation in WME medium, the slices started to show sprouts that increased progressively in number and length on the following days. The results demonstrated that 3 mg/mL of collagen allowed the best visualization of the outgrowth, in which the length of the sprout and the space between sprouts were clearly visible allowing good quantification during prolonged incubation (Fig. 4). However, we experienced difficulties with the immunostaining of the sprouts because of the dense collagen surrounding the slices. Therefore, since the outgrowth was also observed in the PCLS embedded in collagen 2 mg/mL (Fig. 4), we chose this concentration for further experiments.
Fig. 4 Characterization of cell sprouts in different collagen concentrations. Light microscopic image of the outgrowth from the slices (S) after five days of culture in WME medium, indicated by black arrows. (A) Slices without collagen were used as a control. The collagen matrices were prepared in three different concentrations 1 mg/ml (B), 2 mg/ml (C) and 3 mg/ml (D). Scale bar = 500 µm

Besides optimizing the collagen concentration, we also tested the effect of pH on the firmness of collagen matrix. The firmness was evaluated from the capability of the collagen to keep the slices embedded in the matrix during incubation on a reciprocal shaker. It appeared that the collagen was firmer when higher pH (8.0 instead of 7.0) was used, resulting in a better attachment of the slices in collagen matrix after five days of incubation.
Fig. 5 Immunofluorescence staining of collagen-embedded slices with the endothelial cell marker CD31 (A) and BS-1 lectin (B). Green: CD31; Red: BS-1 lectin; and blue: DAPI. The white arrows indicate the sprouts. Scale bar = 50 µm.

The identification of the outgrowths was carried out by immunofluorescence staining using endothelial-specific markers, i.e. CD31 and BS1 lectin. The CD31 antibody used in this study recognize the cytoplasmic tail of this protein in the endothelial cells, while BS1 lectin stains the luminal side of endothelial cells. The outgrowths were both positively stained using CD31 (Fig. 5A) and BS1 lectin (Fig. 5B). A high-power image of the outgrowths using confocal microscopy is shown in Fig. 6. The picture shows the three-dimensional structure of a newly formed microvessel, positively stained for CD31.

Fig. 6 Confocal image of the sprout from a collagen-embedded PCLS. (A) and (B) show the CD31 (green) and DAPI (blue) positive cells lining the sprout, with the magnification of 200x and 400x, respectively. White arrow indicates the sprout. S= slice. Scale bar = 25 µm.
In order to test the response of the tissue on angiogenic factors, the embedded PCLS were treated with the pro-angiogenic factor VEGF-A and the anti-angiogenic compound sunitinib for five days. We did not observe an influence of 20 ng/ml VEGF-A on the outgrowth of sprouts (Fig. 7). However, the outgrowth on the slices was completely prevented by 0.2 µM sunitinib alone or sunitinib together with VEGF-A (Fig. 7).

Fig. 7 Collagen embedded slices were cultured for five days in WME medium. The slices (S) were untreated (A) or treated with 20 ng/ml VEGF-A (B), 0.2 µM sunitinib (C) and 20 ng/ml VEGF-A + 0.2 µM sunitinib (D). The sprouts were observed in control slices (A) and VEGF-A treated (B) slices, indicated by black arrows. Scale bar = 500 µm.

Since we observed a higher CD31 expression in slices incubated in Reg medium (Fig. 2), we also tested this medium on the embedded PCLS. We observed a large increase in sprouts number in the slices incubated in Reg medium compared to WME medium (Fig. 8A). However, the sprouts did not show the typical branched-tube like structure that was seen with WME as culture medium. Immunofluorescence staining on the sprouts showed that the cells were CD31 negative (data not shown) but alpha smooth muscle actin (αSMA) positive indicating that these cells were fibroblasts (Fig. 8B).
Fig. 8 Light microscopic (A) and confocal (B) image of the collagen-embedded slices (S) incubated in Regenemed® medium. The sprouts are indicated with white arrows. The sprouts were positively stained for αSMA (green) and DAPI (blue) was used to stain the nuclei. Scale bars: (A) = 500 μm; (B) = 25 μm

4. DISCUSSION

In this study, we have successfully developed a new technology using PCLS to evaluate angiogenesis in the fibrotic liver. Three different assays were optimized to provide for a more physiologically relevant ex vivo model for fibrosis-associated angiogenesis. The formation of new blood vessels from pre-existing ones (angiogenesis) can occur in physiological conditions, like liver regeneration, or in pathological settings like cirrhosis and cancer. Although angiogenesis in the liver shares similar features with angiogenesis in other tissues and organs, pathological angiogenesis in liver fibrogenesis is highly affected by liver-specific events and by the cellular environment. For instance, the contribution of the hepatic stellate cells, which are the key-player in liver fibrosis, and the presence of two different microvascular structures (sinusoids lined by fenestrated endothelium and large vessels lined by a continuous one) are the liver-specific factors on fibrosis-associated angiogenesis. The liver-specific factors in fibrosis-associated angiogenesis are the reasons why the available non-hepatic in vitro and ex vivo assays in angiogenesis research are not sufficient to provide for an accurate and reliable model that represents the in vivo situation. In addition, these assays were developed for tumor-related studies which do not resemble the physiological situation in liver fibrogenesis.

At first, rat PCLS cultured for 48 h as a model for the early onset of liver fibrosis were applied to investigate fibrogenesis. In this model, an increased procollagen 1α1 expression after 48h of incubation was reported. In the study described in chapter 4 using mouse and human PCLS, we observed an increased CD31 mRNA expression in parallel with the increase of procollagen 1α1 expression. However, in the present study using rat PCLS, the CD31 mRNA was decreased after 24 h and increased again to its pre-incubation level after 48 h of incubation. This result highlights species differences in angiogenic response upon fibrogenesis in untreated PCLS.
from mouse, rat and man. The lack of angiogenic response during incubation for 48 h of rat PCLS was also supported by the unaltered CD31 expression after sunitinib treatment. However, sunitinib affected the CD31 expression in VEGF-A treated PCLS, whereas it decreased the procollagen 1α1 expression in both untreated and VEGF-A treated PCLS. The latter can be explained by the inhibitory effect of sunitinib on the PDGF receptor β, which is an important receptor in HSC activation. Although the increased procollagen 1α1 expression in the VEGF-A treated PCLS was not significant, we found a significant correlation between the CD31 expression and the procollagen 1α1 expression in VEGF-A treated PCLS. This correlation does not provide proof for a causal correlation between fibrosis and angiogenesis in this model, but it suggests a close relationship between these processes. However, based on the increased CD31 mRNA expression we cannot discriminate between extra formation of endothelial cells or increased expression on the existing endothelial cells.

Therefore, we evaluated the CD31 expression at the protein level in slices after five days of incubation, where fibrosis was more extended. Slices were incubated for five days in a standard medium (WME) and in an enriched medium (Reg). The slices remained viable after five days based on unchanged ATP levels, although necrotic zones were observed in the inner cell layers of Reg-incubated slices. An increased protein expression of Collagen I and III was observed in the five-day incubated slices, together with an increased vimentin, desmin, and αSMA staining. In the present study, the results show a significantly increased number of CD31 positive cells and an increased intensity of CD31 staining in the slices incubated in Reg but not in WME. The increased CD31 expression in Reg-incubated slices was observed in the outer cell layers, where an increased collagen deposition was found. Although there were no significant differences in the expression of the fibrosis marker between the slices incubated in WME and Reg, the collagen deposition in both slices was located in different areas. In WME-incubated slices, the collagen was predominantly expressed in areas with bile duct proliferation, while in Reg-incubated slices, collagen deposition was distributed in the parenchyma in the outer cell layers. In addition, it was demonstrated in the previous study that slices incubated in Reg developed hypoxic regions inside the slices due to the thickening of the slices that might block the oxygen penetration. In liver fibrogenesis, structural and anatomical changes have been proposed as factors that affect liver perfusion and compromise oxygen supply. As a consequence, neovascularization is initiated to compensate for the shortage of oxygen supply. Hypoxia is an important stimulus of angiogenesis by stimulating the production of VEGF through hypoxia-inducible factor-1α (HIF-1α) signaling pathway. In liver fibrosis, HIF-1α is known to have an important role in the activation and proliferation of HSCs by regulating different signaling pathways.
Besides CD31, we also tested the expression of another endothelial cell marker, SE-1, which is specific for liver sinusoidal endothelial cells and is correlated with the presence of fenestrae\(^4\). The SE-1 expression was diminished after five days of incubation, indicating that the fenestrae are absent in the new blood vessels. This is in line with the loss of sinusoidal fenestration observed in liver fibrosis in vivo. Thus, although SE-1 cannot be used as marker to evaluate angiogenesis, our finding supports this model as a tool to study liver fibrosis. A further study is required to support the use of this five-day incubated PCLS as model to investigate fibrosis-associated angiogenesis, for instance by adding fibrosis stimulating compounds and test the effect on angiogenesis.

Although we have shown that it was possible to quantify blood vessels in the slices, the technique is relatively time consuming including sample preparation, staining, imaging and image analysis. Therefore, we developed a third assay based on the widely-used aortic ring assay, using PCLS instead of aorta rings to generate liver-specific angiogenic sprouts. This assay provides for a real-time evaluation of the angiogenic response in the slices. In the aortic ring assay, a rat or mouse aorta was embedded in a three-dimensional culture system, which was constructed from extracellular matrix components. There are three different matrices that are commonly used in this aortic ring assay, i.e. fibrin, Matrigel, and type I collagen. In a preliminary study, we tested Matrigel and type I collagen for slice embedding and we observed that Matrigel was not suitable for our system because the sprouts mostly consisted of fibroblasts (data not shown). Therefore, we continued the experiments using type I collagen matrix. In aortic ring assays, 1-1.5 mg/mL collagen was commonly used to generate a collagen matrix with the addition of NaOH. However, this concentration was not sufficient to keep the slices embedded in the matrix because the slices are incubated on a reciprocal shaker to generate a homogenous oxygen distribution. Therefore, the concentration used in this assay was increased to 2 mg/mL, thereby increasing the firmness of the matrix\(^5\). Higher concentration of collagen resulted in problems in the immunohistochemical staining and imaging. Using 2 mg/mL, CD31 positive cells sprouted into the collagen, indicating that these cells are endothelial cells, thereby confirming that this model can be used to study angiogenesis ex vivo.

In order to verify the response of collagen-embedded slices as model for angiogenesis, we tested the effect of pro- and anti-angiogenic compounds and different media on the production of the sprouts. The addition to the pro-angiogenic factor VEGF-A at a concentration of 20 ng/mL did not alter the number or the length of the sprouts. Either a concentration of 20 ng/mL was too low in collagen-embedded slices or endogenous VEGF production was already sufficient to induce maximum sprouting. Interestingly, slices that were treated with sunitinib did not generate any sprout, which indicates that the angiogenic process was fully inhibited. As in the five-day cultured
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PCLS incubation in Reg caused significantly increased blood vessel numbers compared to WME-incubated medium, we investigated the effect of Reg medium on the sprouting. The results show that the cells appearing in the collagen after incubation in Reg medium were all positive for αSMA expression and did not express CD31, which indicates that the sprouts only consisted of fibroblasts. The outgrowths consisting of single cells, many of which are likely to be fibroblasts, are not considered to be microvessel sprouts. This result was not in line with our finding of increased number of blood vessels in Reg-incubated slices. The only difference between both assays is the collagen matrix that surrounded the slices. Since the information of the medium composition is not disclosed, we are not able to explain the difference.

To summarize, the first assay, using rat PCLS incubated for 48 h, may be used for rapid screening of pro- or anti-angiogenic compounds, but it does not allow definitive conclusions on the formation of new blood vessels since the increased CD31 mRNA expression may also mean that the resident endothelial cells are activated. The second assay, PCLS cultured for five days in Reg medium, can show increased formation of CD31 positive cells as well as increased CD31 staining intensity, allowing discrimination between new formation and activation of endothelial cells, as well as characterization of the fenestration of the new blood vessels. Although promising, the effect of fibrotic and angiogenic compounds on CD31 protein expression has not been tested yet and this assay is relatively time consuming. The sprouting assay showed the possibility of generating liver-specific angiogenic outgrowth which can be inhibited by an anti-angiogenic compound. Future experiments should further verify this model and show whether the formation of sprouts is linked to fibrosis in the slices. In the future, we will also test the effect of adding profibrotic proteins, such as transforming growth factor β (TGF-β) on the sprouting formation.

In conclusion, the developed assays in this study allow us to evaluate fibrosis-associated angiogenesis. The response of the model towards pro- and anti-angiogenic factors, the parallel increase in angiogenesis along with the fibrosis, and the evidence of microvessel sprouting indicate that this model can be used to investigate liver-specific angiogenesis. This model can be expanded to the use of human PCLS, which will allow a better translation to the human situation. Finally, this model will contribute to the reduction, replacement and refinement of animal experiments by avoiding in vivo animal experiments with serious discomfort.

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