CELL-SPECIFIC DELIVERY OF INTERFERON ALPHA: IN VIVO EVALUATION

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ABSTRACT
Hepatocytes are often regarded as “victims” and “bystanders” in liver fibrogenesis, but the role of hepatocytes in liver fibrosis is under-examined. Hepatocytes in normal and cirrhotic livers express the pro-angiogenic vascular endothelial growth factor A (VEGF-A) and the anti-angiogenic factor thrombospondin-1 (THBS-1) that might be involved in liver fibrogenesis. In order to unravel the role of hepatocytes in liver fibrosis, we aimed to interfere with the angiogenic balance regulated by hepatocytes through delivering interferon alpha (IFNα) to the hepatocytes. In vitro using HepG2 cells, we have previously shown that our construct, galactose-PEG-IFNα (GPI), was recognized by the asialoglycoprotein receptor (ASGPR) and has similar antiangiogenic effects as native IFNα. In the present study, we aimed to deliver GPI to the hepatocytes in vivo and to test its effect on angiogenesis in a fibrosis model of CCl₄-exposed mice with native IFNα used as positive control. Moreover, we aimed to get more insight in the role of angiogenesis in fibrosis. The localization of the GPI in the liver was detected by immunohistochemical staining with an IFNα recognizing antibody. The results showed that GPI was not only found in hepatocytes, but also in other cell types (endothelial cells and (myo)fibroblasts) in the liver and no difference was observed between the distribution of GPI and native IFNα. This suggests the presence of the IFNα receptor (IFNAR) on other liver cells besides hepatocytes in the mouse. In the current study, both IFNα and GPI decreased the CD31 and VEGF receptor 2 (VEGFR2) expressions, suggesting inhibition of angiogenesis by the lower dose, but not by the higher dose. In addition, an increased VEGF-A expression was found in parallel with this angiogenesis inhibition possibly indicating a feedback mechanism. The angiogenesis inhibition did not affect the liver fibrogenesis in this model, as expression of collagen remained unaltered.

In conclusion, in mice with liver fibrosis, IFNα treatment inhibited angiogenesis without affecting fibrosis. However, since we did not observe a difference in the cellular distribution of GPI and IFNα in the fibrotic liver, we cannot conclude on the role of hepatocytes in this angiogenesis inhibition. In this study, we also did not observe an effect of IFNα on fibrogenesis, despite a decrease in angiogenesis, suggesting that angiogenesis has no direct role in fibrogenesis but may occur as the consequence of increased profibrotic factors that also have an angiogenic effect.
1. INTRODUCTION

Hepatocytes comprise ~85% of the liver mass and have an important role in most of the functions of the liver, such as protein synthesis, glucose metabolism, fat digestion, drug metabolism and bile production. Hepatocytes are also prone to several injuries, including toxic, metabolic, and viral insults. Persistent injury can result in chronic inflammation and wound healing response and can lead to liver fibrogenesis. Hepatocytes are often regarded as “victims” and “bystanders” in liver fibrogenesis, however, it cannot be excluded that they also play a role in fibrogenesis. Recently, hepatocytes have been proposed to contribute to liver fibrosis through a process of epithelial to mesenchymal transition, but this issue remains controversial.

One of the characteristics in liver fibrosis is the existence of abnormal angioarchitecture due to the formation of new blood vessels, known as angiogenesis. Angiogenesis is tightly controlled by the balance of angiogenic activators and angiogenic inhibitors. Angiogenesis factors in the liver are produced by different cell types, one cell type can produce more than one angiogenic factor and each angiogenic factor can be produced by more than one cell type. Hepatocytes in normal and cirrhotic livers express several important pro- and anti-angiogenic factors, i.e. vascular endothelial growth factor A (VEGF-A) and thrombospondin 1 (THBS-1). Therefore, we hypothesized that hepatocytes play role in liver fibrogenesis through angiogenesis.

We aimed to unravel the role of hepatocytes in liver fibrogenesis by interfering with the balance of pro- and anti-angiogenic factors produced by the hepatocytes, through delivery of an anti-angiogenic compound specifically to the hepatocytes. In this study, interferon alpha (IFNα) was used to inhibit angiogenesis because it has been shown in previous studies that IFNα can inhibit endothelial cell migration and proliferation and decrease the secretion of the pro-angiogenic factor VEGF in many human cancer cells. IFNα has been used for many years as a therapeutic cytokine in Hepatitis C treatment, hemangioma and cancer therapy. However, IFNα therapy is associated with a lack of therapeutic specificity due to the presence of interferon alpha receptors (IFNAR) in most of the tissues in the body. In the liver, IFNAR is expressed by hepatocytes and there is no specific report yet on the expression of IFNAR by other cell types in the liver.

In chapter 2, we designed an IFNα construct with galactose-polyethylene glycol in order to specifically deliver it to the asialoglycoprotein receptor (ASGPR), which is expressed abundantly on hepatocytes and recognizes and internalizes galactosylated proteins. We designed this construct by coupling galactose-polyethylene glycol (gal-PEG) moieties to IFNα to enhance uptake in the hepatocytes. In the in vitro study, this modification of IFNα did not affect its biological activity and this construct had similar anti-angiogenic properties as native IFNα. In the current
study, we administered this construct, gal-PEG-IFNα (GPI), to mice with CCl₄-induced liver fibrosis to evaluate its distribution in the liver in vivo and its effect on angiogenesis and fibrosis.

2. MATERIALS AND METHODS

2.1 Synthesis of gal-PEG-IFNα (GPI)
IFNα (Jena Biosciences, Jena, Germany) was coupled to galactose-polyethylene glycol-succinimidyl carboxymethyl (galactose-PEG-SCM) ester (5 kDa, Jenkem Technology, Beijing, China) according to the procedure described in chapter 2.

2.2 CCl₄-exposed liver fibrosis mouse model
Liver fibrosis was induced in six different groups of Balb/c mice (20-22g; Harlan). One group consisted of healthy mice (n=6) and the other five groups were exposed to CCl₄ twice weekly by intra-peritoneal injections with increasing doses (week 1: 0.5 mL/kg; week 2: 0.8 mL/kg and week 3-4: 1 mL/kg of a solution of 0.25 mL CCl₄/mL olive oil, resulting in doses of 0.125, 0.2 and 0.25 mL/kg respectively) for 4 weeks. In week 3 and 4, mice were treated intravenously with 100 µL phosphate-buffered saline (PBS) (n=5), IFNα 400 ng/mL/day (n=4), IFNα 2000 ng/mL/day (n=6), GPI 400 ng/day (n=5), and GPI 2000 ng/day (n=6) three times per week. The dose of GPI refers to the amount of IFNα in the construct. All groups were sacrificed at the end of week 4, blood and livers were collected for further analysis. To investigate the cellular distribution of IFNα and GPI in the liver, a single dose (100 µL) of 4000 ng/mL was injected intravenously 20 min before sacrifice. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen.

2.3 Immunohistochemical staining
To determine the distribution of IFNα and GPI and the protein expression of CD31 and collagen I, immunohistochemical staining was performed on cryostat liver sections (4 µm). Sections were fixed with acetone, rehydrated with PBS and incubated with the primary antibody in appropriate dilution, i.e rabbit polyclonal IFNα antibody (1:600, Thermo Scientific, Rockford, USA), rat monoclonal CD31 antibody (1:200, BD Pharmingen, San Jose, USA) or rabbit polyclonal collagen I antibody (1:50, Rockland, USA) for 1 h at room temperature. The sections were washed with PBS three times and further incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:100, DAKO, Belgium), followed by HRP-conjugated tertiary antibody (1:100, DAKO, Belgium), each for 30min. Peroxidase activity was developed with ImmPACT™ NovaRED™ (Vector Laboratories, Burlingame, USA) for IFNα staining and 3-amino-9-ethyl carbazole (Sigma,
USA) for CD31 and collagen I staining. Subsequently, the nuclei were counterstained with haematoxylin (Fluka Chemie, Buchs, Switzerland). 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 collagen I positive pixels (red) to the total area of pixels in percentage.

Double staining was performed with red staining using NovaRed for IFNα staining to locate the injected compounds and blue staining using BCIP/NBT (Vector Laboratories, Burlingame, USA) for CD31 and goat polyclonal desmin antibody (1:100, Santa Cruz Biotechnology, USA) to identify (myo)fibroblasts.

2.4 Real-time quantitative RT-PCR

The mRNA expression of angiogenesis and fibrosis markers in the mice liver was analyzed using real-time RT-PCR. Liver samples were snap frozen and total RNA was isolated using the Maxwell® 16 LEV SimplyRNA Tissue Kit (Promega, Leiden, The Netherlands). The amount of isolated RNA was measured with an ND-1000 spectrophotometer (Fisher Scientific, Landsmeer, the Netherlands). Reverse transcription of RNA to cDNA was performed using Reverse Transcription System (Promega, Leiden, The Netherlands) 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 level of each marker was measured using the SYBR Green Mastermix (GC Biotech, Alphen aan de Rijn, The Netherlands) and the following primers (50 µM):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procollagen 1α1</td>
<td>TGACTGGAAGAGCCGGAGGT</td>
<td>ATCCATCGGTCACTGCTTCTCT</td>
</tr>
<tr>
<td>CD31</td>
<td>TCCCTGGGAGGGTCTCCAT</td>
<td>GAACAGCCAGCGGGGTTTA</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>GTTCCCGAAGACCTGAGGAG</td>
<td>CTGTGACAGCTCTGATGAAAGC</td>
</tr>
<tr>
<td>VEGFR2/KDR</td>
<td>CAGGACTGAAAGCAGCCAGACTGT</td>
<td>TCAGGCACAGACTCTCTCTCC</td>
</tr>
<tr>
<td>b-actin</td>
<td>ATCGTGCGGTACATCAAAGA</td>
<td>ATGCCACAGGATTCCATACC</td>
</tr>
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The PCR reaction was performed using a 7900HT Real Time PCR (Applied Biosystems) with 1 cycle of 10 min at 95 °C and 40 cycles of 15 secs at 95 °C and 25 secs at 60 °C with a dissociation stage at the end (95 °, 15 secs; 60 °C, 15 secs; 95 °C, 15 secs). Ct values were corrected for the Ct values of the housekeeping gene b-actin. The Ct value of b-actin was similar in all experimental groups.
2.5 ELISA
Liver homogenates were prepared in cold RIPA buffer (10 mL of RIPA buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% Igepal in 0.5% sodiumdeoxycholaat, 0.1% sodium dodecyl sulphate (SDS), and 1 tablet of protease inhibitor) and sonicated for 90 secs. The lysates were centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatants were stored at -80 °C until use. Protein expression of VEGF-A in the liver was determined by using the Murine VEGF Mini ABTS ELISA Development Kit (Peprotech, London, UK) with a small modification. Instead of using 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate provided by the kit, 3,3',5,5'- Tetramethylbenzidine (TMB) substrate (R&D Systems, Minneapolis, USA) was used. Color development was monitored with an ELISA plate reader at 450 nm wavelength and correction set at 550 nm. The calculated concentrations of VEGF-A in the liver homogenates were normalized with the total protein content of the liver homogenate, which was measured using Lowry (BioRad, Veenendaal, The Netherlands).

2.6 Western Blot
Liver homogenates were prepared as described for the ELISA. Homogenates containing 500 µg of protein were mixed with an equal amount of loading buffer (62.5 mM Tris/HCl pH 6.8, 10% glycerol, 2% SDS, 1.55% dithiothreitol and 0.0125% bromophenol blue) and boiled at 95 °C for 5 min. From each sample, 100 µg of protein was applied on SDS-polyacrylamide gel (7.5%). The gel was run by applying 150 V and subsequently transferred to polyvinyldene fluoride (PVDF) membranes. The membranes were blocked for 1h in tris-buffered saline with 0.1 % Tween 20 (TBST) containing 5% non-fat dried milk (BioRad, Veenendaal, The Netherlands) and were incubated with mouse monoclonal THBS-1 antibody (1:500, Thermo Fisher Scientific, Rockford, USA) or rabbit monoclonal VEGFR2 antibody (1:1000, Cell Signaling Technology, Danvers, USA) or mouse monoclonal b-actin antibody (1:10000, Sigma, Saint Louis, USA) overnight at 4 °C. The membranes were further washed with TBST and incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:2000, DAKO, Belgium). The membranes were washed with TBST for 30 min and with tris-buffered saline (TBS) for another 30 min. The protein bands were visualized using VISIGLO™ HRP Chemiluminescent Substrate Kit (Amresco, Solon, USA).

2.7 Statistics
The results were analyzed with One Way ANOVA followed with Dunnett’s Multiple Comparison using GraphPad Prism 7. Data are presented as mean ± standard error mean (SEM). A p-value < 0.05 was considered significant.
3. RESULTS

CCl₄ exposure for 4 weeks reduced the body weight of the mice and treatment with either IFNα or GPI did not alter this reduction (Fig. 1A). White blood cell counts were highly increased by CCl₄ treatment and a significant reduction was observed in mice treated with the lower dose of GPI compared to untreated fibrotic mice, but not with the high dose of GPI and both doses of native IFNα (Fig. 1B). The level of aspartate aminotransferase (AST) and alanine transaminase (ALT) in the blood were used as an indicator of liver injury, and were both increased in response to CCl₄. Mice treated with the lower dose of IFNα showed a higher AST level compared to untreated fibrotic mice (Fig. 1C). This effect, however, was not observed at the higher dose and no difference was observed in the ALT level in the mice treated with either IFNα or GPI (Fig. 1D).

*Fig. 1 Effects of IFNα and GPI on body weight and white blood cell count in control and CCl₄-exposed mice. Fibrosis induction with CCl₄ for 4 weeks reduced mice body weight (A) and increased white blood cell (WBC) count (B). Neither IFNα nor GPI altered the body weight reduction, but treatment with 40 ng of GPI significantly decreased the WBC in CCl₄-exposed mice. (C) Significant increase in liver enzyme aspartate aminotransferases (AST) was observed in mice treated with 40 ng of IFNα but no effect was observed on ALT level (D). *p<0.05 compared to PBS-treated CCl₄ mice. Data are presented as means (±SEM). n=4-6

Immunohistochemical staining of IFNα in fibrotic livers of mice treated with IFNα or GPI showed that there was no difference in the distribution of native IFNα and GPI (Fig. 2A and 2B respectively). The staining showed that the location of both compounds was not specific for one cell type, but IFNα staining was found in the endothelial cells of the portal veins, arteries and...
central veins, and was most prominent in the fibrotic septa but absent in the bile ducts (Fig. 2A, B). The staining was not found in the livers of animals that did not receive IFNα or GPI (data not shown). Moreover, staining was also observed in hepatocytes as cytoplasmatic staining and in some areas, the basolateral membrane was positive (Fig. 2C). Double staining with CD31 as endothelial cell marker and with desmin as (myo)fibroblasts marker confirmed the co-localization of both compounds in the CD31 positive endothelial cells (Fig. 2D) and desmin positive (myo)fibroblasts (Fig. 2E).

![Fig. 2](image)

**Fig. 2** Distribution of IFNα (A) and GPI (B) in the CCl₄-exposed mice liver. The positive staining (red) shows the location of respectively IFNα and GPI. There was no difference in the distribution of both compounds, the staining was found in the endothelial cells of the portal vein (pv), artery (a), and central vein (cv), staining was prominent in the fibrotic septa (f) and absent in the bile ducts. The staining of GPI in the cytoplasm and basolateral membrane of the hepatocytes is indicated with the black arrow in (C). The red arrow in (D) shows the co-localization of the GPI (red) in CD31 positive cells (blue). While the blue arrow in (E) shows the co-localization of the GPI (red) in desmin positive cells (blue). Pictures in (C, D, E) are similar for IFNα (not shown) Scale bars = 300 µm (top figures) and 50 µm (bottom figures).

CD31, the endothelial cell marker, was used as a surrogate marker for angiogenesis. In healthy mice, the CD31 staining was located on larger veins and arteries with faint staining along the sinusoids (Fig. 3A1). In CCl₄-exposed mice, the CD31 protein expression was strongly increased and this CD31 staining was prominent in the fibrotic septa in addition to the vascular and
sinusoidal endothelial localization indicating angiogenesis (Fig. 3A2). VE-cadherin staining showed similar pattern to the CD31 staining in the liver of healthy and fibrotic mice (data not shown). Treatment of fibrotic mice with the lower dose of IFNα and GPI reduced the CD31 protein expression (Fig. 3A3, 3A5, 3B), confirming the anti-angiogenic effect of IFNα but the higher dose of both compounds did not affect the CD31 protein expression significantly (Fig. 3A4, 3A6, 3B). Remarkably, the CD31 mRNA expression was similar in control and fibrotic livers and no effect was observed in mice treated with either IFNα or GPI (Fig. 3C).

**Fig. 3** Effect of IFNα and GPI on the level of the endothelial cell marker (CD31) in CCl4-exposed mice. (A) In healthy controls, strong CD31 staining was observed in veins and arteries and faint staining was seen in the sinusoids (A1). A strong CD31 staining was visible in fibrotic septa (A2) in PBS-treated CCl4 mice in addition to the vascular and sinusoidal endothelial staining, which was suppressed by the low dose of both IFNα (A3) and GPI (A5) but not by the high dose (A4, A6). The results of the quantification CD31 protein expression are given in (B) as percentage of stained area. Fibrosis induction with CCl4 did not alter the mRNA expression of CD31 (C) (mRNA levels of CD31 in all groups were normalized with b-actin as housekeeping gene).

*p<0.05 compared to PBS-treated CCl4 mice. Data are presented as means (±SEM). n= 4-6

Scale bars = 200 µm

Our previous *in vitro* study showed the effects of IFNα and GPI on VEGF-A and THBS-1 in HepG2 cells. Both compounds showed anti-angiogenic effects by decreasing the protein...
expression of the pro-angiogenic factor VEGF-A and increasing the expression of the anti-angiogenic factor THBS-1. In contrast, in the fibrotic liver in vivo both compounds increased the pro-angiogenic VEGF-A protein expression without any effect on the mRNA expression (Fig. 4A, 4B). In addition, the untreated CCl4-exposed mice showed a lower VEGF-A mRNA and protein expression (Fig. 4A, 4B) compared to the healthy controls. Moreover, the THBS-1 expression was reduced in CCl4 treated animals compared to healthy animals, but was not affected by either IFNα or GPI at mRNA (data not shown) and protein level (Fig. 4C).

**Fig. 4** Effect of IFNα and GPI on the expression of angiogenic factors in CCl4-exposed mice. Fibrosis induction with CCl4 decreased the mRNA (A) and protein (B) expression levels of the pro-angiogenic factor VEGF-A in the liver. VEGF-A protein expression was determined with ELISA and expressed relative to the total protein in the liver. Treatment with 40 ng IFNα and GPI increased VEGF-A protein expression. (C) Western blot analysis of THBS-1 and VEGFR2; b-actin was used as loading control. Along with a decreased VEGF-A expression in CCl4-exposed mice, the protein (D) and mRNA (E) expressions of its receptor (VEGFR2) were also decreased. Treatment with IFNα and GPI (40 ng) further reduced the VEGFR2 protein expression, but its mRNA expression remained unchanged.

* p<0.05 compared to PBS-treated CCl4 mice. Data are presented as means (±SEM). n=4-6

VEGF-A binds to VEGF receptor 1 and 2 (VEGFR1 and VEGFR2) but exerts its angiogenic effect only through VEGFR2. In the current study, fibrotic mice treated with the lower dose
of IFNα and GPI had lower protein expression of VEGFR2 compared to untreated fibrotic mice (Fig. 4C, D). This result is consistent with the effect of both IFNα and GPI at lower dose on CD31 and VEGF-A protein expressions. However, we did not observe any effect of the treatment on VEGFR2 expression at mRNA level (Fig. 4E).

**Fig. 5** Effects of IFNα and GPI on the levels of fibrosis in CCl4-exposed mice. Both IFNα and GPI did not affect the mRNA levels of procollagen 1α1 (C) and the protein levels of collagen I (D) in CCl4-exposed mice. (E) Photomicrographs of representative collagen I staining (red) in mice livers.

*p<0.05 compared to PBS-treated CCl4 mice. Data are presented as means (±SEM). n=4-6

Scale bars = 300 µm

Lastly, we investigated the effect of IFNα and GPI on liver fibrogenesis. Both IFNα and GPI showed no effect on the mRNA expression of procollagen 1α1 and on the collagen I protein expression compared to untreated fibrotic mice (Fig. 5C, D, E). Similar results were obtained for the expression of alpha smooth muscle actin (data not shown).
4. DISCUSSION

In this study, we aimed to target IFN\(\alpha\) to the hepatocytes of mice with CCl\(_4\)-induced liver fibrosis by coupling it to galactose-PEG. However, the in vivo results show that there was no difference in cellular distribution of IFN\(\alpha\) and GPI in the liver. Both compounds showed binding to endothelial cells, (myo)fibroblasts and hepatocytes and no difference in the relative distribution among these cell types was observed between IFN\(\alpha\) and GPI. The galactose ligand coupled to the IFN\(\alpha\) was hypothesized to drive the GPI to the ASGPR expressed by hepatocytes. Both IFN\(\alpha\) and GPI induced STAT1 phosphorylation in HepG2 cells, which in case of GPI could be inhibited by lactosylated HSA, suggesting the involvement of the ASGPR to the effect of GPI (chapter 2). In the current in vivo study, no such targeting to the ASGPR on hepatocytes could be observed from the immunohistochemical data.

The lack of targeting of GPI to the hepatocytes in vivo might be due to the fact that binding of both IFN\(\alpha\) and GPI to the IFNAR is much higher on the endothelial cells, (myo)fibroblasts and hepatocytes, than binding to the ASGPR. As a consequence, the binding to the ASGPR on the hepatocytes is negligible compared to the binding to the IFNAR. IFNAR is a common receptor for type I interferons including IFN\(\alpha\) and consists of two subunits, a low- (IFNAR1) and a high-affinity (IFNAR2) receptor component. IFNAR1 and IFNAR2 form a heteromeric complex upon binding and activation by type I interferons. The distribution of IFNAR on hepatocytes has been previously reported. Although there has not been any study yet showing the distribution of IFNAR expression in the liver other than hepatocytes, the reported IFNAR expression in human umbilical vein endothelial cells and cardiac fibroblasts suggests that IFNAR might be also expressed by endothelial cells and fibroblasts in the liver. Indeed, our histochemical results for the first time indicate the binding of IFN\(\alpha\) in vascular and sinusoidal endothelial cells, (myo)fibroblasts and hepatocytes of the fibrotic mouse liver, which is similar to that of GPI, suggesting the presence of IFNAR in these cells, thereby supporting our hypothesis on the lack of effect of targeting of IFN\(\alpha\).

Another explanation is that GPI might bind to scavenger receptors expressed by endothelial cells, macrophages and hepatic stellate cells. Binding of gal-PEG to IFN\(\alpha\) increases its negative charge and it is known that negatively charged proteins are substrates for scavenger receptor-mediated internalization. This increased binding of GPI compared to IFN\(\alpha\) to the scavenger receptor could have counteracted the binding to the ASGPR on the hepatocytes. However, we did not observe a difference in the binding of GPI and IFN\(\alpha\). Thus, we think that the scavenger receptor is less likely to influence the binding of GPI compared to the IFNAR. The third explanation is the internalization of GPI by ASGPR might degrade the compound, thereafter
cannot be detected by immunohistochemical staining. Whether the compound was rapidly internalized and degraded in hepatocytes remains to be tested. The ASGPR is still present on hepatocytes in fibrotic mice (data not shown).

In order to test whether coupling to gal-PEG would reduce the systemic immunosuppressive effect of IFNα, WBC count was assessed as an inflammation marker. The increased WBC count in the mice exposed to CCl₄ indicates the inflammation that accompanies fibrosis. A decrease of WBC count was found in fibrotic mice treated with the lower dose of GPI, which might indicate less inflammation, although intrahepatic TNFα level was not reduced (data not shown). Nevertheless, a decrease of WBC count might also indicate IFNα-mediated bone marrow suppression, due to the presence of IFNAR in the bone marrow. However, the effect on the WBC count was not observed in mice treated with native IFNα. This might be due to the higher systemic exposure to GPI compared to IFNα, as it is well known that pegylation can cause prolongation of the half-life in the blood circulation, as was shown by Bansal et al. for IFNγ coupled to 5kD PEG. Of note, their results also showed that PEG-IFNγ decreased the WBC count and there was no difference in WBC count when IFNγ was coupled to PEG of different size. The result in our study showed that the conjugation of galactose to PEG apparently does not reduce the systemic effect of IFNα.

To investigate the effect of GPI on angiogenesis, we used CD31 as marker for endothelial cells. Angiogenesis emerges in parallel with liver fibrogenesis. Indeed, immunohistochemical staining showed an increased CD31 protein expression that was observed in the sinusoids in the fibrotic septa in fibrotic mice. Surprisingly, in this experiment we did not observe a difference in CD31 mRNA expression between healthy mice and CCl₄-exposed mice, indicating upregulation at the posttranslational level. Treatment with the lower dose of IFNα and GPI reduced the CD31 protein expression significantly, indicating indeed an anti-angiogenic effect. The effect, however, was not observed with the higher dose of both IFNα and GPI. Such a U-shaped dose-response was also found in our in vitro study on the effect of IFNα and GPI using HepG2 cells and HUVEC. A U-shaped dose response was also observed for the anti-angiogenic effects of IFNα in human bladder cancer. In addition, several other molecules, used for therapeutic targeting of angiogenesis in cancer, have been shown to display hormesis with bell-shaped, U-shaped or J-shaped dose-response curves.

VEGF-A is also well known as marker of angiogenesis because an increase in VEGF-A expression is correlated with the progression of angiogenesis. However, we observed a different result in our study. Compared to healthy mice, the mice that were exposed with CCl₄ had a decreased VEGF-A expression in parallel with the increased CD31 protein expression. This
The decrease of VEGF-A expression in fibrotic mice was also observed by Kantari-Mimoun et al., in mice that were exposed with CCl₄ for 12 weeks. When the mice were left untreated for 4 weeks (recovery period), the VEGF-A expression increased again in parallel with a decrease of fibrosis markers²⁸,²⁹.

The increase of VEGF-A expression after treatment of fibrotic mice with free IFNα or GPI is inconsistent with the result from the findings with HepG2 cells in chapter 2, which showed a decrease of VEGF-A expression upon exposure to IFNα and GPI. This discrepancy between the results in HepG2 cells and in the fibrotic mouse livers could indicate a species difference. However, it is always difficult to extrapolate in vitro data in cell lines to the in vivo situation during disease progression. Moreover, we have strong indications that the effect of IFNα in the liver is an overall result of the effect in several different cell types in addition to that in hepatocytes. An anti-angiogenic effect that is accompanied by an increased VEGF-A expression was also observed in a study using cilengitide, an inhibitor of integrin αvβ3 and αvβ5, to assess its anti-angiogenic effect in liver fibrosis. Cilengitide inhibited angiogenesis in bile duct ligation and thioacetamide-induced liver fibrosis in the rat but aggravated liver fibrogenesis⁴⁰. The increased VEGF-A expression along with decreased CD31 expression could indicate a feedback mechanism due to hypoxia. The lack of supplying blood vessels because of the angiogenesis inhibition can compromise oxygen delivery to the liver parenchyma⁴⁰. As a consequence, the expression of hypoxia-inducible proteins is increased, including the expression of VEGF-A⁴⁰. Interestingly, a decreased VEGFR2 expression was also observed after IFNα treatment. This decreased VEGFR2 expression might be the reason why the increased VEGF-A expression did not result in increased angiogenesis. In addition to VEGF-A, we also investigated the effect on THBS-1 to explain the anti-angiogenic effect observed in this study, but we did not find any difference in the expression of THBS-1 at mRNA and protein level. A possible explanation of the effect of IFNα and GPI on angiogenesis in our study is a direct effect on endothelial cells, assuming that IFNAR is present in these cells, which is supported by the immunohistochemical localization¹⁹,²⁵.

Lastly, one of the aims of this study was to evaluate the role of angiogenesis in liver fibrosis. In this study, we did not observe any effect on the fibrosis markers upon angiogenesis inhibition. We are not the first to observe this. In the study conducted by Kantari-Mimoun et al., genetic ablation of VEGF in scar-infiltrating myeloid cells inhibited angiogenesis in 12-week CCl₄-exposed mice but did not affect liver fibrogenesis³⁸. Although our immunohistochemical studies suggest that the IFNAR is expressed by the HSC, it seemed that treatment with IFNα or GPI did not influence the liver fibrogenesis.
In conclusion, we have successfully inhibited angiogenesis in CCl₄-exposed mice with IFNα and GPI. However, since we did not observe a difference in the cellular distribution of both compounds in the fibrotic liver, we cannot conclude on the role of hepatocytes in this angiogenesis inhibition. In this study, we also did not observe an effect of IFNα on fibrogenesis, despite a decrease in angiogenesis, suggesting that angiogenesis has no direct role in fibrogenesis but may occur as the consequence of increased profibrotic factors that also have an angiogenic effect.

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REFERENCES


28. van der Sluijs P, Bootsma HP, Postema B, Moolenaar F, Meijer DKF (1986) Drug targeting to the liver with lactosylated albumins: Does the glycoprotein target the drug or is the drug targeting the glycoprotein?. Hepatology 6:723-728


adult volunteers. Hepatogastroenterology 43:301-305


