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IN FOCUS

Coagulation status modulates murine hepatic fibrogenesis: implications for the development of novel therapies

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Summary. *Background:* There is strong evidence demonstrating that coagulation system activation contributes to wound healing and promotes organ fibrosis. Several epidemiological studies have now shown that prothrombotic status, including carriage of the factor (F)V Leiden mutation, is associated with rapid progression of hepatic fibrosis. *Objectives:* To assess the effect of a procoagulant state on progression of hepatic fibrosis in a controlled environment and to test whether anticoagulation could attenuate fibrogenesis. *Methods:* We investigated the effects of coagulation status on liver fibrosis development in a mouse model of chronic toxic liver injury. Prothrombotic FV Leiden mutant mice, C57BL/6 control animals and anticoagulated mice were studied after chronic exposure to carbon tetrachloride. *Results:* Carriage of the FV Leiden mutation caused a significant increase in hepatic fibrosis. Anticoagulation with warfarin significantly reduced fibrosis progression in wild-type mice but was less effective against the profibrotic FV Leiden mutation. Changes in the fibrosis scores were mirrored by changes in liver hydroxyproline content and hepatic stellate cell activation detected by α-smooth muscle actin expression. *Conclusions:* These results demonstrate that coagulation status has a strong influence on hepatic fibrogenesis. It is likely that thrombin signaling through the proteinase-activated receptor 1 (PAR_1) receptor expressed on hepatic stellate cells is responsible for this relationship. These results represent the first reported use of anticoagulation to slow hepatic fibrogenesis and suggest a potential novel anti-fibrotic therapeutic approach for the future.

Keywords: anticoagulation, factor V Leiden, fibrosis, hepatitis, liver, PAR_1.

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Introduction

Hepatic fibrosis results from a sustained wound healing response to chronic liver injury [1]. Unchecked, stellate cell mediated collagen accumulation within the hepatic parenchyma proceeds to cirrhosis. Existing therapy is targeted at the etiology of hepatic fibrosis. If this proves unsuccessful, there are currently no effective therapeutic interventions to slow the rate of fibrogenesis.

Hypercoagulability plays an important but under-recognized role in many aspects of liver disease [2]. Early evidence of a role for coagulation in the pathogenesis of liver disease came from studies examining the effects of murine hepatitis virus infection where microthombi were demonstrated within the hepatic microvasculature [3,4]. Wanless *et al*. subsequently reported similar findings in patients with cirrhosis [5,6] and epidemiological studies identified an association between host prothrombotic status and rapid progression of hepatic fibrosis [7–9]. Under normal physiological conditions, thrombin catalyses the conversion of fibrinogen to fibrin, which then mediates clot formation. Thrombin has a well-recognized role in the coagulation cascade and in activating platelet aggregation. In addition, it is also recognized to act as an activator of hepatic stellate cells, the mediators of hepatic fibrogenesis, via proteinase-activated receptor 1 (PAR_1). Increased thrombin generation may thus elevate levels of PAR_1 signaling and promote hepatic fibrogenesis.

As part of a negative feedback loop, thrombin activates Protein C (APC) which in turn cleaves Factor V at the R506 primary cleavage site [10,11]. The factor (F)V Leiden mutation is a single-base pair substitution conferring an amino acid change from Arginine to Glutamine in codon 506. This abolishes the primary APC cleavage site, leaves factor V activity unchecked and hence confers a procoagulant tendency. This common mutation affects 1–8.5% of the Caucasian population and predisposes to thromboembolic disease [12].

Using the carbon tetrachloride-induced liver injury model [13], we sought first to establish whether carriage of the FV Leiden mutation (as an exemplar of pro-thrombotic tendency)

could indeed influence progression of hepatic fibrosis. Second, we tested whether anticoagulation with warfarin (coumarin) could ameliorate the fibrosis progression in the same model. Both male and female animals were studied to acertain whether the gender effect observed in the original epidemiological studies persisted in a laboratory environment [7].

Materials and methods

Animals studied and carbon tetrachloride exposure

Transgenic FV Leiden mice generated by site-directed mutagenesis were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a congenic C57BL/6 background (Strain B6.129S2-F5^{tm2Dgi}/J) [14]. The ΔR504Q mutation carried by these mice is homologous to the human mutation. Homozygous carriage of the FV Leiden mutation was confirmed by genotyping [14]. FV Leiden and wild-type C57BL/6 mice (Harlan, Bicester, UK) aged 6–8 weeks were used in this study (group sizes in Table 2). Animals were housed under standard conditions. All research was approved by the local ethical review process and carried out in accordance with the Animal (Scientific Procedures) Act 1986 taking care to minimize any distress caused to the animals.

Based on effective induction of fibrosis in pilot studies, mice were treated with carbon tetrachloride (CCl_4) diluted in a corn oil vehicle and administered by intraperitoneal injection on alternate week days. The dose of CCl_4 administered was increased weekly in a stepwise fashion (0.125, 0.25, 0.5, 1 mL kg^{-1} body weight) with the maximum dose maintained from day 21.

Warfarin was administered to anticoagulate mice by adding the drug to the drinking water. A warfarin dose of 1 μg mL^{-1} was chosen as pilot studies indicated that this approximately doubles the whole blood clotting time but minimizes spontaneous adverse events.

Histopathology and digital image analysis

Tissue collected at each time point was fixed in 10% formalin and processed into paraffin wax. Sections were stained with Hematoxylin & Eosin (H&E) or Chromotrope Aniline Blue (CAB) trichrome to delineate fibrosis. Samples were examined by two histopathologists, blinded to which study group each sample was from, and a joint score agreed using an adapted Ishak Modified Histological Activity Index [15,16] (Table 1). Sections were also stained using the collagen-specific stain Pico-Sirius Red before undergoing digital image analysis to determine the mean percentage area of fibrosis. An average of 12 fields (10× objective) throughout each section were captured using a Zeiss AxioVert 200M microscope and analysed using AnalySIS software (Olympus Soft Imaging Solutions, Watford, UK). Tissue from a subset of male animals culled at the 4-week time point was also immunohistochemically stained for a marker of hepatic stellate cell activation, alpha-Smooth Muscle Actin (primary antibody Clone 1A4; Dako UK Ltd, Ely, UK) [17].

Table 1 Modified histological scoring system for quantifying liver fibrosis

Score	Description
0	No fibrosis
1	Fibrous expansion (spurs) around some central veins
2	Fibrous expansion around > 50% central veins
3	Fibrous expansion around most central veins plus some CV-to-CV bridging
4	Fibrous expansion around most central veins plus marked (> 50%) CV-to-CV bridging
5	Marked bridging fibrosis plus some nodules
6	Cirrhosis (> 50% nodularity)

Total RNA extraction and quantification of relative gene expression by RT-PCR

The effects of coagulation status were most marked in male animals culled at the 4-week time point and so this group was selected for further detailed analysis of stellate cell activation, collagen deposition and gene induction.

Total RNA was extracted from snap frozen liver tissue (RNeasy Mini kit, Qiagen Ltd, Crawley, UK), reverse transcribed (RETROscript kit; Ambion Inc, Austin, TX, USA) and quantitative polymerase chain reaction (PCR) analysis performed in triplicate using TaqMan Gene Expression Assay reagents (Applied Biosystems). Primers specific for Pro-collagen type I (Col1a2, Mm00483888_m1) were studied. Data were normalized to the endogenous housekeeping gene Hypoxanthine Guanine Phosphoribosyl Transferase (*hprt*) and fold change differences in expression relative to non-warfarin treated C57BL/6 control animals calculated using the Comparative $\Delta\Delta C_T$ method [18].

Comparative Western Blot analysis of alpha-smooth muscle actin (αSMA) protein expression

Expression of αSMA is a sensitive marker of hepatic stellate cell activation to a fibroblast-like phenotype therefore αSMA levels were measured as an indicator of Hepatic Stellate Cell (HSC) activation. Protein was extracted from snap frozen liver tissue as previously described [19]. Fifty micrograms of protein pooled from each study group was loaded onto a 4–12% NuPage MOPS gel (Invitrogen Ltd, Paisley, UK) and a Western blot performed onto a nitrocellulose membrane. αSMA was labeled with a primary mouse monoclonal antibody (Clone 1A4, Dako UK Ltd) and chromogenic detection performed (Western Breeze, Invitrogen). Gel images were captured and comparative quantification of protein performed using Image-J software (US NIH, Bethesda, MD, USA).

Tissue hydroxyproline measurement

Collagen helices deposited within the liver during fibrogenesis are stabilized by hydroxyproline. This amino acid is almost exclusively confined to collagenous connective tissue and so may be used as a surrogate to quantify collagen deposition [20].

Liver hydroxyproline content was determined using a colorimetric technique as previously described [21]. Samples were tested in triplicate compared with standards of known hydroxyproline concentration and results expressed as μg hydroxyproline per gram of liver tissue.

Lipid peroxidation malondialdehyde (MDA) assay

CCl_4 induces tissue damage by release of free radicals and reactive oxygen species as it is metabolized by cytochrome P450 [22]. Tissue oxidative stress is reflected by the MDA content [23]. To determine if warfarin interacts with CCl_4 metabolism and thus limits its injurious effect, hepatic MDA content was measured. Liver tissue from cohorts of warfarin-treated and non-warfarin-treated C57BL/6 mice culled 24 h after their last CCl_4 exposure ($n = 5$ per group) was analysed using a colorimetric MDA assay (Lipid Peroxidation Assay #437634, Calbiochem, USA).

Statistical analysis

Statistical analysis performed using SPSS v12 (SPSS Inc., Chicago, IL, USA). Normally distributed continuous variables were compared using Student's *t*-test; results are represented as mean (±SEM). Ordinal and non-normally distributed variables were tested using the non-parametric Mann–Whitney *U*-test. Statistical significance was accepted at $P < 0.05$.

Results

At the start of the experiment, FV Leiden mice weighed on average 23.5 ± 0.3 g and control mice weighed 21.1 ± 0.2 g, CCl_4 doses were body weight adjusted as described above. No fibrosis was observed in warfarin-treated and non-treated animals that had not been exposed to carbon tetrachloride. In warfarin-treated animals, whole blood clotting time was 8.5 ± 2 min (mean ± standard deviation) compared with 4.5 ± 0.6 min in control animals. Prothrombin time, measured in subgroup of animals during the study, was 24.6 ± 1.5 s in warfarin-treated vs 11.3 ± 0.15 s in non-warfarin-treated mice. No differences in warfarin response between mutant and non-mutant mice were observed. Early mortality (ill health necessitating cull of animal in accordance with regulatory requirements) was limited to three warfarin-treated animals where death was as a result of hemorrhage soon after intraperitoneal injection.

Lipid peroxidation malondialdehyde (MDA) assay

No significant difference in hepatic MDA content was observed between warfarin-treated and untreated C57BL/6 mice 24 h after exposure to CCl_4 (mean MDA concentration ± SEM: warfarin treated animals 0.24 ± 0.03 μmoles mg^{-1}; control mice 0.26 ± 0.02 μmoles mg^{-1}). We therefore conclude that effects of anticoagulation described below are not an artefact as a result of a reduction in the injurious capacity of CCl_4.

Fibrosis progression in wild-type C57BL/6, FV Leiden and anticoagulated mice

Representative histological images are shown in Fig. 1. Histopathology and DIA scores are presented in Table 2 with intergroup analysis summarized in Table 3 and Supplementary Fig. S1. Animals culled at the start of the experiment had no evidence of spontaneous hepatic fibrosis (score = 0, C57BL/6 $n = 5$, FvL $n = 4$).

After 2 weeks of CCl_4 exposure, FV Leiden mice exhibited bridging fibrosis and occasional nodule formation (Fig. 1C). In contrast, C57BL/6 control mice showed milder fibrosis with evidence of fibrous expansion of peri-venous areas and minimal bridging (Fig. 1B). Warfarin-treated mice of both strains had lower fibrosis scores than the corresponding mice given CCl_4 without warfarin but the differences did not reach statistical significance at this stage (C57BL/6 shown in Fig. 1A). Digital analysis (presented in Table 2) supports these observations. Mean fibrosis area was increased by 80% in male FV Leiden mutants compared with C57BL/6 controls ($P = 0.002$) and more than doubled in female mice ($P = 0.007$).

Non-warfarin treated C57BL/6 control mice culled at 4 weeks exhibited greater hepatic fibrosis than those culled at 2 weeks; however, progression was much more pronounced in FV Leiden mutant mice. After 4 weeks of CCl_4 exposure, FV Leiden mutant mice had developed extensive bridging fibrosis with nodule formation and in some cases established cirrhosis (Fig. 1F,I). Numerically this is a 60% increase in mean fibrosis area over C57BL/6 controls for FV Leiden males, $P = 0.003$ and a 74% increase for females, $P < 0.001$. Table 2 also shows that FV Leiden and the non-warfarin treated C57BL/6 male mice have significantly higher mean histology scores than female littermates (C57BL/6 Δ ± SEM 0.86 ± 0.28, $P = 0.012$; FvL 0.71 ± 0.28, $P = 0.023$). Warfarin administration reversed this gender effect. Warfarin-treated male C57BL/6 mice showed only moderate fibrous expansion around the central veins (Fig. 1D,G) with a mean reduction in histological score of 2.25 ± 0.38 (SEM) compared with control ($P = 0.007$).

DIA demonstrated that mean fibrosis area was 33% reduced in male warfarin-treated C57BL/6 mice (Table 2, $P = 0.005$). There was no significant difference in fibrosis area between warfarin-treated C57BL/6 mice at 2 and 4 weeks (Δ 0.44% ± 0.27%, $P = 0.171$) although control animals had a 1.0% ± 0.22% increase ($P = 0.01$). At 4 weeks, the effects of warfarin were less marked in females with no significant differences from control detected. FV Leiden mice treated with warfarin showed levels of fibrosis similar to FV Leiden mice which had not been anticoagulated (Table 2).

Tissue hydroxyproline content

Carriage of the FV Leiden mutation in males was associated with a significant 49% increase in tissue hydroxyproline content over control (FV Leiden 215.6 ± 27.4 μg g^{-1} liver tissue vs. C57BL/6 146.6 ± 23.7 μg g^{-1}, $P = 0.043$). Warfarin treatment of C57BL/6 mice was associated with a 32%

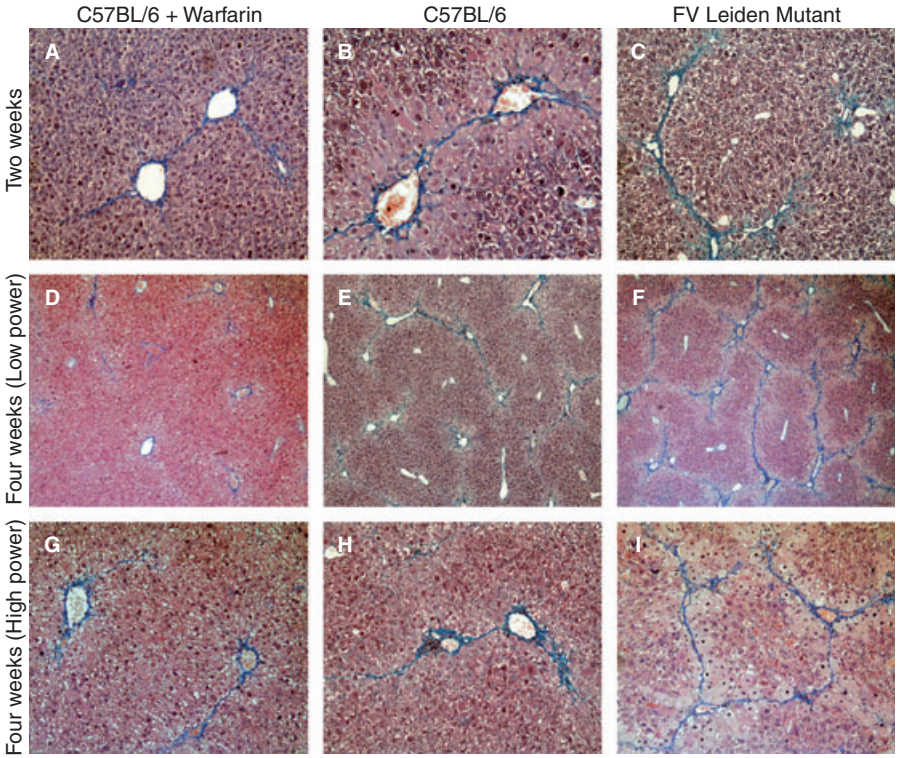

Fig. 1. Representative histological images. Liver tissue sections were stained with Chromotrope Aniline Blue (CAB) trichrome to deliniate fibrosis. A, B and C: Warfarin-treated (A) and non-warfarin-treated (B) C57BL/6 mice after 2 weeks of CCl_4 exposure. Factor (F)V Leiden mutant tissue (C) showing evidence of bridging fibrosis and early nodule formation after 2 weeks CCl_4 exposure. Original magnification ×300. D, E and F:Tissue from a warfarin-treated C57BL/6 mouse (D) with evidence of fibrous spur formation but no nodel formation. Tissue from a non-warfarin-treated C57BL/6 mouse (E) with evidence of bridging fibrosis. FV Leiden mutant tissue (F) showing nodule formation approaching established cirrhosis. Original magnification ×150. G, H and I: High magnification images of CAB Trichrome-stained liver tissue from a warfarin-treated C57BL/6 mouse (G), a non-warfarin treated C57BL/6 animal (H) and an FV Leiden mutant mouse (I). Original magnification × 300**.**

Table 2 Effect of CCl_4 on histology and digital image analysis scores for each study group

		Histology Score (maximum 6)				Mean percentage area fibrosis			
		2 Weeks		4 Weeks		2 Weeks		4 Weeks	
Study group	Gender	Mean (SEM)	*n*	Mean (SEM)	*n*	Mean (SEM)	*n*	Mean (SEM)	*n*
C57BL/6	M	3.00 (0.45)	6	4.00 (0.26)	6	1.76 (0.2)	6	2.76 (0.09)	6
	F	2.00 (0.32)	5	3.14 (0.14)	7	1.40 (0.29)	5	2.38 (0.37)	7
FV Leiden	M	4.11 (0.2)	9	4.86 (0.26)	7	3.18 (0.28)	9	4.47 (0.4)	7
	F	2.78 (0.15)	9	4.15 (0.15)	13	2.97 (0.32)	9	4.14 (0.21)	13
C57BL/6 + Warfarin	M	2.60 (0.4)	5	1.75 (0.25)	4	1.43 (0.11)	5	1.84 (0.27)	4
	F	2.40 (0.25)	5	2.88 (0.29)	8	1.89 (0.27)	5	2.11 (0.14)	8
FV Leiden + Warfarin	M	3.83 (0.48)	6	4.57 (0.2)	7	2.68 (0.27)	6	3.82 (0.18)	6
	F	3.40 (0.4)	5	4.17 (0.31)	6	2.92 (0.33)	5	3.70 (0.35)	6

reduction in hydroxyproline content although this did not reach statistical significance (C57BL/6 + Warfarin 98.4 ± 18.3 μg g^{-1}, $P = 0.243$). Warfarin did not have a significant effect on hydroxyproline content in FV Leiden mice.

Hepatic gene expression

Expression of genes that reflect hepatic fibrogenic activity was measured using quantitative RT-PCR. Data are presented in Fig. 2 as fold change relative to the C57BL/6 control group and SEM range. Pro-collagen type 1 expression was significantly increased by 32% in mice carrying the FV Leiden mutation (1.32, 1.02–1.67, $P = 0.027$) and reduced by 40% (0.6, 0.45–0.82, $P = 0.001$) in C57BL/6 animals treated with warfarin.

Relative quantification of alpha-Smooth muscle actin protein expression

Western blot analysis demonstrated that the level of αSMA expression in male FV Leiden mice after 4 weeks CCl_4 exposure was approximately twice that of C57BL/6 controls

Table 3 Differences in mean percentage area fibrosis and mean histology score following 2 or 4 weeks CCl_4 exposure

		Male Mean difference relative to C57BL/6				Female Mean difference relative to C57BL/6			
		2 Weeks		4 Weeks		2 Weeks		4 Weeks	
Study group	Variable	Mean (SEM)	*P*-value	Mean (SEM)	*P*-value	Mean (SEM)	*P*-value	Mean (SEM)	*P*-value
FV Leiden	% Area Fib.*	1.42 (0.38)	0.002	1.71 (0.45)	0.003	1.57 (0.48)	0.007	1.76 (0.39)	0.000
	Hist. Score†	1.11 (0.44)	0.031	0.86 (0.37)	0.044	0.78 (0.30)	0.034	1.01 (0.24)	0.001
C57BL/6 + Warfarin	% Area Fib.*	−0.33 (0.24)	0.207	−0.92 (0.24)	0.005	0.49 (0.39)	0.252	−0.27 (0.37)	0.480
	Hist. Score†	−0.40 (0.61)	0.492	−2.25 (0.38)	0.007	0.40 (0.40)	0.339	−0.27 (0.34)	0.429
		Mean difference relative to FV Leiden				Mean difference relative to FV Leiden			
FV Leiden + Warfarin	% Area Fib.*	0.50 (0.41)	0.238	0.65 (0.47)	0.196	0.05 (0.5)	0.921	0.44 (0.39)	0.275
	Hist. Score†	0.28 (0.46)	0.746	0.29 (0.33)	0.424	−0.62 (0.43)	0.101	−0.01 (0.31)	0.918

P-values derived from Student's *t*-test (*) or Mann–Whitney *U*-test (†).

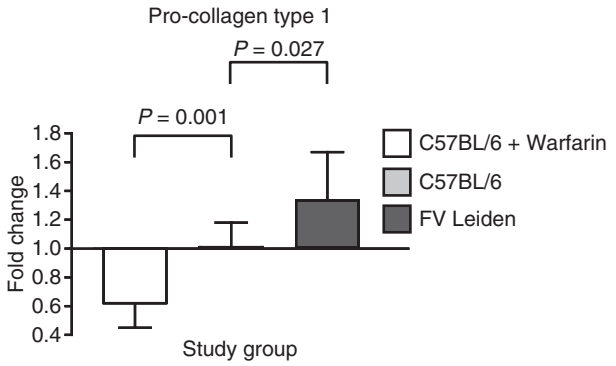

Fig. 2. Differential gene expression between study groups relative to C57BL/6. Pro-collagen type 1 was analysed by quantitative RT-PCR (n = 4–7 mice per group). Normalized fold change values relative to C57BL/6 are shown ± SEM. Pro-collagen type 1 expression was significantly reduced in C57BL/6 mice treated with warfarin and increased in factor (F)V Leiden mutant mice.

(Fig. 3). Treatment with warfarin reduced αSMA expression in both C57BL/6 and FV Leiden animals by 10–12%, respectively. As illustrated in Fig. 4, immunohistochemical staining of a subset of tissue samples supported these observations. An

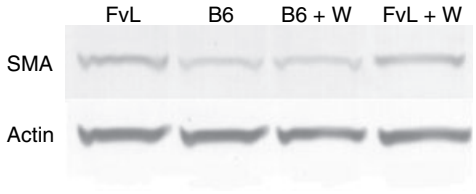

Fig. 3. Western blot of alpha-smooth muscle actin protein expression. Image of Western blot stained with primary antibodies to αSMA and skeletal Actin (control). The corrected area under the optical density curve of each band was measured using Image J software (NIH, Bethesda, MD, USA) and is shown as a fold change relative to C57BL/6 mice [factor (F)V Leiden 2.1, C57BL/6 1.0, C57BL/6 + Warfarin 0.9, FV Leiden + Warfarin 1.8). After 4 weeks of CCl_4 exposure, FV Leiden mutant mice have greater αSMA expression, and hence hepatic stellate cell activation, than C57BL/6 mice. Anticoagulation was shown to reduce αSMA expression. (n = 4–7 mice per group).

increase in the number of αSMA positive cells was seen in FV Leiden mice while a reduction was seen in warfarin-treated C57BL/6 animals. These findings are consistent with increased stellate cell activation in response to CCl_4 exposure in animals that carry the FV Leiden homozygote mutation and reduced activation in those concomitantly treated with warfarin (Fig. 4).

Discussion

The data presented here supports the original observations in chronic viral hepatitis that carriage of procoagulant mutations, such as the FV Leiden mutation, confers an accelerated hepatic fibrosis phenotype during chronic liver injury [7–9]. The effect of mutation carriage was apparent in subjects of either gender after 2 weeks and became more marked at the 4-week time point. The histological demonstration of increased fibrous tissue deposition was corroborated by demonstrably greater tissue hydroxyproline content and higher levels of Pro-collagen type 1 gene expression. The association between prothrombotic status and tissue fibrosis is also pertinent to fibrogenesis in other organ systems. Indeed, corroborative data has been reported by investigators studying pulmonary fibrosis where prothrombotic status, and specifically carriage of the FV Leiden mutation, has been shown to lead to more rapid development of fibrosis in mice after Bleomycin inhalation [24]. Reports describing enhanced collagen accumulation leading to the development of fibrotic skin lesions in tissue plasminogen activator deficient mice have also been published [25] and the role of plasminogen activator inhibitor-1 (PAI-1) in tissue fibrogenesis has been described in several pathologies including renal, hepatic, cardiovascular and pulmonary disease [26].

The reduction in fibrosis observed in animals treated with warfarin suggests that anticoagulation retards fibrogenesis. This was most evident in male C57BL/6 mice whereas FV Leiden mice appeared to be resistant to warfarin. The dose of warfarin used in these experiments was limited by risk of hemorrhage and may have been insufficient to overcome the

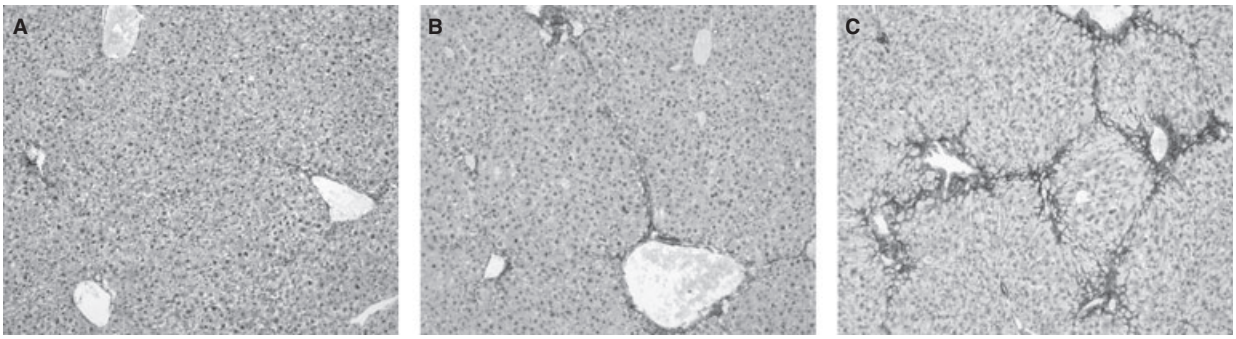

Fig. 4. Immunohistochemical staining of liver tissue for alpha-smooth muscle actin. Tissue samples from some male animals culled at 4 weeks were stained for αSMA. Relative to C57BL/6 control mice (B), these demonstrated reduced numbers of αSMA positive cells in warfarin-treated C57BL/6 mice (A) and increased numbers of αSMA positive cells in factor (F)V Leiden mutant animals (C).

pro-thrombotic and pro-coagulant effect of the homozygous carriage of the FV Leiden mutation. In human populations only the effect of heterozygote FV Leiden carriage has been studied and we aim to study FV Leiden heterozygote mice in the future. Interestingly the direct thrombin inhibitor, Ximelagatran, appears to have been sufficiently potent to overcome the effect of the FV Leiden mutation without observable risk of adverse events in the mice (Q.M. Anstee and M.R. Thursz, unpublished data). While this study represents the first reported therapeutic use of coumarin-based anticoagulation to slow hepatic fibrogenesis, similar results have been reported with low molecular weight heparin [27] and dipyridamole [28] in other models of liver damage and with PAR_1 knockout mice, aerosolized heparin and urokinase in pulmonary fibrosis [29,30]. There is also a precedent for the use of anticoagulation to treat pulmonary fibrosis in humans, exemplified in a trial of warfarin anticoagulation in patients with idiopathic pulmonary fibrosis in which mortality was reduced by 50% [31].

The interaction of gender on the rate of fibrosis has previously been observed in human Hepatitis C (HCV) studies where, as demonstrated here, it is recognized that males progress more rapidly [32,33]. Evidence suggests that estrogen acts as an *in vivo* antioxidant, inhibits stellate cell profibrotic TGFβ expression and possibly reduces platelet-derived growth factor (PDGF)-induced cell proliferation [34,35]. While an effect of anticoagulation on fibrogenesis was hypothesized, the significant differences in response to anticoagulation between the genders were an unexpected finding. While a full explanation for this effect is not yet available, recently published data suggests that female sex hormones are able to modulate PAR_1 expression in certain tissues [36] and so may blunt the contribution of this pathway to stellate cell activation. Our findings are consistent with previous reports that the association between FV Leiden mutation carriage and accelerated hepatic fibrosis with HCV infection was most pronounced in men [7] and may explain in part the failure to identify an effect of HCV carriage on fibrosis rate in an all female patient cohort [37]. Further, in humans an increased risk of recurrent thromboembolic events is seen in males [38]. Taken together these findings suggest that males may be more sensitive to APC resistance and the generation of thrombin [38] and that the effects of coagulation status may therefore be of greater significance in males. As a result of these phenotypic differences, subsequent detailed study of fibrogenesis was confined to male animals culled at the 4-week time point.

The mechanism through which coagulation status influences hepatic fibrosis is not fully elucidated. Some have proposed that micro-thrombi disrupt the flow of blood within the hepatic parenchyma and so lead to tissue ischemia, parenchymal extinction and fibrosis [5,6]. PAR_1 (thrombin) receptor expression has been shown to be upregulated during acute and chronic tissue injury [39]. PAR_1 receptors are present on hepatic stellate cells which, when activated, deposit fibrous matrix. Hepatic stellate cells may be activated directly by thrombin [40] or by pro-inflammatory cytokines secreted by macrophages and platelets (which also express PAR_1). In support of PAR_1-mediated fibrosis acceleration, it has been shown that PAR_1 antagonists ameliorate fibrosis in an animal model [41] and that polymorphisms in the PAR_1 gene influence the rate of disease progression in HCV infection [42].

In conclusion, these data provide support for the hypothesis that coagulation status promotes hepatic stellate cell activation and enhances hepatic fibrogenesis. Alongside the clinical response to warfarin therapy in pulmonary fibrosis, these results provide a rational for testing anticoagulants as a treatment for liver fibrosis where the cause of the underlying liver disease cannot be removed.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supplementary material

The following supplementary material can be found at http://www.blackwell-synergy.com/loi/jth :

Fig. S1. Degree of hepatic fibrosis by histological score and digital analysis at 4 weeks.
This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2008.03015.x
(This link will take you to the article abstract).

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References

1 Friedman SL. Liver fibrosis - from bench to bedside. *J Hepatol* 2003; **38**(Suppl. 1): S38–53.
2 Northup PG, Sundaram V, Fallon MB, Reddy KR, Balogun RA, Sanyal AJ, Anstee QM, Hoffman MR, Ikura Y, Caldwell SH. Hypercoagulation and thrombophilia in liver disease. *J Thromb Haemost* 2007; **6**: 2–9.
3 Levy GA, MacPhee PJ, Fung LS, Fisher MM, Rappaport AM. The effect of mouse hepatitis virus infection on the microcirculation of the liver. *Hepatology* 1983; **3**: 964–73.
4 MacPhee PJ, Schmidt EE, Keown PA, Groom AC. Microcirculatory changes in livers of mice infected with murine hepatitis virus. Evidence from microcorrosion casts and measurements of red cell velocity. *Microvasc Res* 1988; **36**: 140–9.
5 Wanless IR, Wong F, Blendis LM, Greig P, Heathcote EJ, Levy G. Hepatic and portal vein thrombosis in cirrhosis: possible role in development of parenchymal extinction and portal hypertension. *Hepatology* 1995; **21**: 1238–47.
6 Wanless IR, Liu JJ, Butany J. Role of thrombosis in the pathogenesis of congestive hepatic fibrosis (cardiac cirrhosis). *Hepatology* 1995; **21**: 1232–7.
7 Wright M, Goldin R, Hellier S, Knapp S, Frodsham A, Hennig B, Hill A, Apple R, Cheng S, Thomas H, Thursz M. Factor V Leiden polymorphism and the rate of fibrosis development in chronic hepatitis C virus infection. *Gut* 2003; **52**: 1206–10.
8 Poujol-Robert A, Boelle PY, Poupon R, Robert A. Factor V Leiden as a risk factor for cirrhosis in chronic hepatitis C. *Hepatology* 2004; **39**: 1174–5.
9 Papatheodoridis GV, Papakonstantinou E, Andrioti E, Cholongitas E, Petraki K, Kontopoulou I, Hadziyannis SJ. Thrombotic risk factors and extent of liver fibrosis in chronic viral hepatitis. *Gut* 2003; **52**: 404–9.
10 Suzuki K, Nishioka J, Hashimoto S. Protein C inhibitor. Purification from human plasma and characterization. *J Biol Chem* 1983; **258**: 163–8.
11 Luckow EA, Lyons DA, Ridgeway TM, Esmon CT, Laue TM. Interaction of clotting factor V heavy chain with prothrombin and prethrombin 1 and role of activated protein C in regulating this interaction: analysis by analytical ultracentrifugation. *Biochemistry* 1989; **28**: 2348–54.
12 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.
13 Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review. *Semin Liver Dis* 1990; **10**: 56–65.
14 Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, Yang TL, Metz AL, Gallagher KP, Tyson JA, Rosenberg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. *Blood* 2000; **96**: 4222–6.
15 Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN, James Phillips M, Portmann BG, Poulsen H, Scheuer PJ, Schmid M, Thaler H. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**: 696–9.
16 Recknagel RO, Glende EA Jr, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther* 1989; **43**: 139–54.
17 Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 1986; **103**: 2787–96.
18 ABI Biosystems Inc. User Bulletin 2 ABI PRISM 7700 Sequence Detection System. Applied Biosystems Inc, 1997 & 2001.
19 Toye AA, Moir L, Hugill A, Bentley L, Quarterman J, Mijat V, Hough T, Goldsworthy M, Haynes A, Hunter AJ, Browne M, Spurr N, Cox RD. A new mouse model of type 2 diabetes, produced by N-ethyl-nitrosourea mutagenesis, is the result of a missense mutation in the glucokinase gene. *Diabetes* 2004; **53**: 1577–83.
20 Lee HS, Shun CT, Chiou LL, Chen CH, Huang GT, Sheu JC. Hydroxyproline content of needle biopsies as an objective measure of liver fibrosis: emphasis on sampling variability. *J Gastroenterol Hepatol* 2005; **20**: 1109–14.
21 Bergman I, Loxley R. Lung tissue hydrolysates: studies of the optimum conditions for the spectrophotometric determination of hydroxyproline. *Analyst* 1969; **94**: 575–84.
22 Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; **33**: 105–36.
23 Moore K, Roberts LJ. Measurement of lipid peroxidation. *Free Radic Res* 1998; **28**: 659–71.
24 Xu Z, Westrick RJ, Shen YC, Eitzman DT. Pulmonary fibrosis is increased in mice carrying the factor V Leiden mutation following bleomycin injury. *Thromb Haemost* 2001; **85**: 441–4.
25 de Giorgio-Miller A, Bottoms S, Laurent G, Carmeliet P, Herrick S. Fibrin-induced skin fibrosis in mice deficient in tissue plasminogen activator. *Am J Pathol* 2005; **167**: 721–32.
26 Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. *J Thromb Haemost* 2005; **3**: 35–45.
27 Abdel-Salam OM, Baiuomy AR, Ameen A, Hassan NS. A study of unfractionated and low molecular weight heparins in a model of cholestatic liver injury in the rat. *Pharmacol Res* 2005; **51**: 59–67.
28 Wanless IR, Belgiorno J, Huet PM. Hepatic sinusoidal fibrosis induced by cholesterol and stilbestrol in the rabbit: 1. Morphology and inhibition of fibrogenesis by dipyridamole. *Hepatology* 1996; **24**: 855–64.
29 Howell DC, Johns RH, Lasky JA, Shan B, Scotton CJ, Laurent GJ, Chambers RC. Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 2005; **166**: 1353–65.
30 Gunther A, Lubke N, Ermert M, Schermuly RT, Weissmann N, Breithecker A, Markart P, Ruppert C, Quanz K, Ermert L, Grimminger F, Seeger W. Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med* 2003; **168**: 1358–65.
31 Kubo H, Nakayama K, Yanai M, Suzuki T, Yamaya M, Watanabe M, Sasaki H. Anticoagulant therapy for idiopathic pulmonary fibrosis. *Chest* 2005; **128**: 1475–82.
32 Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; **349**: 825–32.
33 Poynard T, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis c. *J Hepatol* 2001; **34**: 730–9.

34 Itagaki T, Shimizu I, Cheng X, Yuan Y, Oshio A, Tamaki K, Fukuno H, Honda H, Okamura Y, Ito S. Opposing effects of oestradiol and progesterone on intracellular pathways and activation processes in the oxidative stress induced activation of cultured rat hepatic stellate cells. *Gut* 2005; **54**: 1782–9.

35 Shimizu I. Impact of oestrogens on the progression of liver disease. *Liver Int* 2003; **23**: 63–9.

36 Hague S, Oehler MK, MacKenzie IZ, Bicknell R, Rees MC. Protease activated receptor-1 is down regulated by levonorgestrel in endometrial stromal cells. *Angiogenesis* 2002; **5**: 93–8.

37 Goulding C, O'brien C, Egan H, Hegarty JE, McDonald G, O'farrelly C, White B, Kelleher D, Norris S. The impact of inherited prothrombotic risk factors on individuals chronically infected with hepatitis C virus from a single source. *J Viral Hepat* 2007; **14**: 255–9.

38 Kyrle PA, Minar E, Bialonczyk C, Hirschl M, Weltermann A, Eichinger S. The risk of recurrent venous thromboembolism in men and women. *N Engl J Med* 2004; **350**: 2558–63.

39 Marra F, DeFranco R, Grappone C, Milani S, Pinzani M, Pellegrini G, Laffi G, Gentilini P. Expression of the thrombin receptor in human liver: up-regulation during acute and chronic injury. *Hepatology* 1998; **27**: 462–71.

40 Marra F, Grandaliano G, Valente AJ, Abboud HE. Thrombin stimulates proliferation of liver fat-storing cells and expression of monocyte chemotactic protein-1: potential role in liver injury. *Hepatology* 1995; **22**: 780–7.

41 Fiorucci S, Antonelli E, Distrutti E, Severino B, Fiorentina R, Baldoni M, Caliendo G, Santagada V, Morelli A, Cirino G. PAR1 antagonism protects against experimental liver fibrosis. Role of proteinase receptors in stellate cell activation. *Hepatology* 2004; **39**: 365–75.

42 Martinelli A, Knapp S, Anstee Q, Worku M, Tommasi A, Zucoloto S, Goldin R, Thursz M. Effect of a thrombin receptor (protease-activated receptor 1, PAR-1) gene polymorphism in chronic hepatitis C liver fibrosis. *J Gastroenterol Hepatol* 2007; DOI: 10.1111/j.1440-1746.2007.05220.x.