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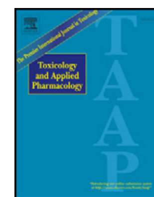

Short Communication

Von Willebrand factor deficiency reduces liver fibrosis in mice

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ABSTRACT

Liver diseases are associated with complex changes in the hemostatic system and elevated levels of the platelet-adhesive protein Von Willebrand factor (VWF) are reported in patients with acute and chronic liver damage. Although elevated levels of VWF are associated with fibrosis in the general population, the role of VWF in acute and chronic liver injury has not been examined in depth in experimental settings. We tested the hypothesis that VWF deficiency inhibits experimental liver injury and fibrosis. Wild-type (WT) and VWF-deficient mice were challenged with carbon tetrachloride (CCl_4) and the impact of VWF deficiency on acute liver injury and chronic liver fibrosis was determined. VWF deficiency did not significantly affect acute CCl_4-induced hepatocellular necrosis in mice. Chronic CCl_4 challenge, twice weekly for 6 weeks, significantly increased hepatic stellate cell activation and collagen deposition in livers of WT mice. Interestingly, hepatic induction of several profibrogenic and stellate cell activation genes was attenuated in VWF-deficient mice. Moreover, birefringent sirius red staining (indicating type I and III collagens) and type I collagen immunofluorescence indicated a reduction in hepatic collagen deposition in CCl_4-exposed VWF-deficient mice compared to CCl_4-exposed WT mice. The results indicate that VWF deficiency attenuates chronic CCl_4-induced liver fibrosis without affecting acute hepatocellular necrosis. The results are the first to demonstrate that VWF deficiency reduces the progression of liver fibrosis, suggesting a mechanistic role of elevated plasma VWF levels in cirrhosis.

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1. Introduction

Complex changes in the hemostatic system are evident in patients with acute and chronic liver injury (Lisman et al., 2010; Tripodi and Mannucci, 2011). Chronic liver disease can result in replacement of functional liver mass with extracellular matrix, primarily collagens. The outcome of this process is cirrhosis, which can predispose patients to thrombosis, and may also contribute to progression of liver disease itself (Jairath and Burroughs, 2013). Among the hemostatic molecules disturbed in both acute and chronic liver disease is Von Willebrand factor (VWF), a glycoprotein that functions to bridge platelets with an exposed collagen surface. Several studies report that plasma levels of VWF (Reuken et al., 2015) are increased in patients with acute liver failure, as well as cirrhosis, with high levels predictive of poor outcome in this patient population (La Mura et al., 2011; Ferlitsch et al., 2012; Hugenholtz et al., 2013; Maieron et al., 2014; Kalambokis et al., 2016). Suggesting a functional connection, VWF levels are associated with the severity of liver fibrosis in the general population (Plompen et al., 2015). Thus, there is strong evidence in patients linking elevated VWF levels with both acute liver injury and with consequences of chronic liver disease such as cirrhosis.

Despite the association between VWF levels and cirrhosis in patients, the precise nature of this association is not understood. Increased plasma VWF levels in cirrhosis are most likely ascribed to endothelial cell activation, induction of hepatic expression (Ferro et al., 1996; Hollestelle et al., 2004), or perhaps reduced liver-mediated clearance (Lisman et al., 2006). That is, elevated plasma VWF levels may be a biomarker of elevated hepatic expression, driven by liver disease itself. However, strong evidence indicates that components of the hemostatic system actually drive the activation of hepatic stellate cells in experimental liver fibrosis (Anstee et al., 2011; Tripodi et al., 2011). Indeed, several studies illustrate the potential for coagulation proteases, both by activation of intracellular signaling and formation of intrahepatic microthrombi, to directly exacerbate liver fibrosis (Anstee et al., 2008; Rullier et al., 2008; Sullivan et al., 2010). Importantly, the association

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of VWF with cirrhosis, while inferred by clinical association studies, has not been examined in experimental studies to determine whether VWF plays a direct mechanistic role in liver fibrosis.

To address this critical gap in the literature, we tested the hypothesis that VWF directly contributes to experimental liver fibrosis by employing an established mouse model of genetic VWF deficiency. Using this tool, we determined the effect of VWF deficiency on acute carbon tetrachloride (CCl_4)-induced liver injury and on liver fibrosis driven by chronic CCl_4 challenge. Collectively, these studies represent the first published report describing the impact of VWF deficiency in experimental settings of acute and chronic liver injury.

2. Materials and methods

2.1. Mice

Congenic C57Bl/6 J $VWF^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME), were maintained by homozygous breeding, and have been described previously (Denis et al., 1998). Age- and sex-matched wild-type C57Bl/6 J mice were purchased from Jackson Laboratory and allowed to acclimate in the same facility for at least 3 weeks prior to experimental challenge. Male and female mice used for these studies were between the ages of 8–14 weeks. Mice were housed in an AAALAC-accredited facility and allowed ad libitum access to purified drinking water and rodent chow. All animal procedures were approved by the Michigan State University IACUC.

2.2. Acute and chronic CCl_4 challenge

10% CCl_4 in corn oil vehicle was administered to mice by intraperitoneal injection (10 ml/kg). For acute liver injury studies, samples were collected 48 or 72 h after CCl_4 challenge. Chronic CCl_4-induced liver fibrosis was induced by administration of CCl_4 every 3–4 days (i.e., every Tuesday and Friday) for 6 weeks. Blood and liver samples were collected three days after the last injection of CCl_4.

2.3. Histopathology, immunohistochemistry and immunofluorescence

Hematoxylin and eosin (H&E), sirius red, alpha-smooth muscle actin (α-SMA), proliferating cell nuclear antigen (PCNA) and type I collagen stains were performed and analyzed as described previously (Sullivan et al., 2010; Joshi et al., 2016; Kopec et al., 2016). Necrosis area was calculated as described previously (Joshi et al., 2016).

2.4. Determination of VWF, lipid peroxidation, and serum ALT

Plasma VWF antigen levels were determined by ELISA as described previously (Groeneveld et al., 2016) and pooled normal human plasma was utilized to develop a standard curve. Thiobarbituric acid reactive substances (TBARS) were determined using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Serum alanine aminotransferase (ALT) activity was determined using commercial reagents (Thermo Fisher, Waltham, MA).

2.5. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR)

Detection and quantification of select mRNAs by SYBR Green qPCR was performed using primers described previously (Joshi et al., 2016).

2.6. Statistical analyses

Comparison of two groups was performed using Student's *t*-test. Comparison of three or more groups was performed using one- or two-way analysis of variance (ANOVA), as appropriate, and Student-Newman-Keul's post hoc test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Effect of VWF deficiency on acute CCl_4-induced liver injury

Plasma VWF levels were significantly increased in CCl_4-exposed wild-type mice at 48 and 72 h (Fig. 1A). Complete VWF deficiency did not affect lipid peroxidation at 48 h, a surrogate marker of CCl_4 metabolism (Fig. 1B). Both male and female mice were challenged with CCl_4 and there was no apparent sex-dependent difference in liver injury, in agreement with prior studies (Bhathal et al., 1983). Serum ALT activity, an indicator of hepatocellular injury, was significantly increased in CCl_4-exposed male and female $VWF^{-/-}$ mice compared to wild-type mice at 48 h (Fig. 1C). However, this did not correspond to a significant increase in centrilobular liver necrosis in CCl_4-exposed $VWF^{-/-}$ mice (Fig. 1D-E). Serum ALT activity and hepatocellular necrosis were reduced at 72 h, regardless of genotype (Fig. 1C-E). Moreover, the number of PCNA-positive hepatocytes at both 48 and 72 h was not significantly affected by VWF deficiency (Fig. 1F-G).

3.2. VWF deficiency reduces α-smooth muscle actin and profibrogenic gene induction in liver after chronic CCl_4 challenge

Plasma VWF levels were significantly increased in wild-type mice after chronic (6 weeks) CCl_4 challenge (Fig. 2A). Serum ALT activity was increased modestly in wild-type mice challenged with chronic CCl_4 and this was unaffected by VWF deficiency (Fig. 2B). As anticipated, chronic CCl_4 challenge in wild-type mice was associated with increased hepatic stellate cell transition to myofibroblasts, marked by significant increases in hepatic *Acta2* and corresponding α-SMA protein expression (Fig. 2C-E). Interestingly, induction of *Acta2* and α-SMA protein was significantly reduced in $VWF^{-/-}$ mice (Fig. 2C-E). In agreement with these measures suggesting reduced stellate cell activation, induction of additional profibrogenic genes, *Tgfb1*, *Tgfb2* and *Col1a1*, was also significantly reduced in CCl_4-exposed $VWF^{-/-}$ mice compared to CCl_4-exposed wild-type mice (Fig. 2F–H).

3.3. VWF deficiency reduces liver fibrosis after chronic CCl_4 challenge

Hepatic stellate cells are well-recognized as the primary cellular source of extracellular matrix (e.g., collagens) in liver fibrosis; thus, we utilized multiple approaches to examine the effect of VWF deficiency on liver fibrosis. First, we quantified hepatic collagen deposition by examining sirius red-stained sections under polarized light, an approach that isolates the birefringent property of type I and III collagen (Junqueira et al., 1978; Street et al., 2014). Collagen deposition significantly increased in CCl_4-challenged wild-type mice, but this increase was significantly reduced in CCl_4-challenged $VWF^{-/-}$ mice (Fig. 3A–B). To validate the reduction in collagen deposition in $VWF^{-/-}$ mice, we evaluated type I collagen deposits by a direct immunolabeling approach. By this method, type I collagen deposits significantly increased in livers of wild-type CCl_4-challenged mice, and this was significantly attenuated in CCl_4-challenged $VWF^{-/-}$ mice (Fig. 3C–D). Altogether, the results indicate that VWF deficiency significantly reduces collagen deposition in a well-validated experimental setting of liver fibrosis.

4. Discussion

Increased plasma VWF levels are closely associated with poor outcome in patients with cirrhosis, and VWF levels are associated with fibrosis in the general population (La Mura et al., 2011; Ferlitsch et al., 2012; Maieron et al., 2014; Plompen et al., 2015; Kalambokis et al., 2016). Building on this clinical association, we provide the first experimental evidence that VWF directly contributes to liver fibrosis. Similar to patients with liver disease, we observed that both acute and chronic experimental liver injury elevated plasma VWF levels. Whereas VWF deficiency had minimal effect on acute CCl_4 hepatotoxicity, activation of hepatic stellate cells, suggested by α-SMA expression, and hepatic

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