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Fibrinogen, acting as a mitogen for tubulointerstitial fibroblasts, promotes renal fibrosis

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Fibrinogen plays an important role in blood coagulation but its function extends far beyond blood clotting being involved in inflammation and repair. Besides these crucial functions it can also promote tissue fibrosis. To determine whether fibrinogen is involved in the development of renal tubulointerstitial fibrosis we utilized the profibrotic model of unilateral ureteral obstruction in fibrinogen-deficient mice. In the heterozygotes, obstruction was associated with a massive deposition of intrarenal fibrinogen. Fibrinogen deficiency provided significant protection from interstitial damage and tubular disruption, attenuated collagen accumulation, and greatly reduced *de novo* expression of α-smooth muscle actin in the obstructed kidney. While no differences were found in renal inflammatory cell infiltration, fibrinogen deficiency was associated with a significant reduction in interstitial cell proliferation, a hallmark of renal fibrosis. *In vitro*, fibrinogen directly stimulated renal fibroblast proliferation in a dose-dependent manner. This mitogenic effect of fibrinogen was mediated by at least three different cell surface receptors on renal fibroblasts: TLR2, TLR4, and ICAM-1. Thus, our study suggests that fibrinogen promotes renal fibrosis by triggering resident fibroblast proliferation.

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The primary role of the coagulation system is to prevent blood loss after vascular injury by ensuring the formation of stable hemostatic clots. However, the function of the coagulation system extends far beyond blood clotting, including such diverse processes as protection against infection, inflammation, and tissue repair.[1,2] Some of these effects have specifically been linked to fibrinogen.[3–5] Fibrinogen is found in plasma at a concentration of 1.5–4.5 g/l and consists of two identical subunits that contain three polypeptide chains: α, β, and γ.[2] Cleavage by thrombin releases fibrinopeptides A and B and converts fibrinogen into fibrin. Activation of the coagulation cascade and the deposition of fibrin into the provisional extracellular matrix is an integral part of tissue injury. The fibrin-containing matrix can promote infiltration of repair cells and can activate healing mechanisms such as angiogenesis, reepithelialization, cell proliferation, and wound contraction.[1] However, if imbalanced, these wound-healing mechanisms can contribute to excessive scar formation and organ fibrosis. Gene-targeting studies suggest that fibrinogen might play a role in tipping the balance between healthy wound-healing and fibrotic scarring.[6] Consistent with this hypothesis, fibrinogen has been demonstrated to promote the development of fibrosis in several organs including pancreas, skin, and muscle.[6–8]

In the kidney, progressive fibrosis is the final pathway of most chronic diseases leading to irreversible loss of renal function.[9] Fibrinogen might be a novel candidate molecule in the development of renal fibrosis given the above-mentioned results from other organs. Massive fibrin deposition is commonly observed in renal biopsies in a variety of acute and chronic kidney diseases,[10–12] and experimentally it has been shown that the lack of fibrinogen can attenuate renal impairment in inflammatory glomerulonephritis.[13] Therefore, the aim of this study was to address the hypothesis that fibrinogen plays a direct role in the development and progression of renal fibrosis.

To test the impact of fibrinogen on renal fibrosis, kidneys from fibrinogen knock-out ($Fib^{-/-}$) and heterozygous ($Fib^{+/-}$) mice were compared at 1 or 2 weeks of unilateral ureteral obstruction (UUO). It was found that the lack of fibrinogen was associated with a significant protection from

developing tubulointerstitial damage. UUO kidneys from $Fib^{-/-}$ mice showed significantly less deposition of extracellular matrix and greatly attenuated expansion of resident fibroblasts. *In vitro*, fibrinogen stimulated renal fibroblast proliferation directly, which was mediated by at least three different cell surface receptors.

RESULTS

Fibrinogen accumulates in UUO kidneys and associates with tubulointerstitial damage

Consistent with published data by Yamaguchi *et al.*,[14] we observed a more than tenfold increase of fibrinogen by immunoblot in UUO kidneys from wild-type mice after 7 days of ureteral obstruction (Figure 1a). Immunoreactivity recognizing both fibrinogen and fibrin was restricted to the intravascular space in contralateral kidneys, whereas it was massively enhanced in the tubulointerstitial space of UUO kidneys (Figure 1b and c). To test whether fibrinogen would contribute to the development of renal fibrosis, $Fib^{-/-}$ mice were subjected to UUO and compared with heterozygous littermates. $Fib^{-/-}$ mice had a normal phenotype under baseline conditions and no appreciable increase in bleeding risk or healing defects after UUO surgery. At day 14 of obstruction, kidneys from $Fib^{+/-}$ animals exhibited fibrinogen deposits throughout the entire renal interstitium (Figure 1d and e). In some areas we found particularly intense accumulations, which was often associated with increased tubulointerstitial damage (Figure 1d). As expected, we found no fibrinogen in kidneys from $Fib^{-/-}$ mice (Figure 1f). At day 7 of UUO, both groups showed dilated tubules with flattened epithelium and a mild increase in interstitial volume without histologically appreciable differences (Figure 1g–i). At 14 days, however, loss of tubular structures and expansion of interstitial cells and matrix was significantly more severe in $Fib^{+/-}$ when compared with $Fib^{-/-}$ mice (Figure 1i–k). Whereas tubules were often replaced by fibrous tissue in $Fib^{+/-}$ kidneys, tubular structures persisted significantly longer in $Fib^{-/-}$ mice (Figure 1j–m). We consistently found a significant reduction in intrarenal collagen deposition in $Fib^{-/-}$ mice as shown by Picrosirius Red staining (Figure 2a and b) and immunoblot (Figure 2c).

Fibrinogen deficiency has no impact on inflammatory cell infiltration

Although their role is not completely understood,[15] inflammatory cells infiltrating the interstitium are characteristic features of renal fibrosis. Consistently, increased numbers of infiltrating CD45-positive leukocytes, CD4-positive lymphocytes, F4/80-positive macrophages, and Ly-6B.2-positive polymorphonuclear cells were found in UUO kidneys of both groups. Fibrinogen deficiency however, had no impact on the respective cell counts (Figure 2d and e).

Fibrinogen deficiency is associated with reduced numbers of (myo)fibroblasts in UUO

α-Smooth muscle actin (α-SMA), which is used as a marker of myofibroblasts,[9] was significantly upregulated in the tubulointerstitial space of UUO kidneys from both groups. However, the upregulation was significantly higher in $Fib^{+/-}$ animals when compared with $Fib^{-/-}$ littermates (Figure 3a and b). For further analysis of differences in interstitial cell expansion, immunostainings using S100A4 were performed, showing a massive increase in S100A4-positive cells in $Fib^{+/-}$ when compared with $Fib^{-/-}$ kidneys (Supplementary Figure S1B online). S100A4 is widely used as a marker for fibroblasts and myofibroblasts,[16] but its specificity has been questioned because of expression by inflammatory cells.[17] We therefore quantified only those S100A4-positive cells that were CD45 negative (Figure 3c–e), and found that fibrinogen deficiency was associated with significantly lower numbers of S100A4+/CD45− cells after 7 and 14 days of UUO (Figure 3e). These results were further confirmed by using ER-TR7, an independent fibroblast marker[18] (Figure 3f and g), and suggest a differential expansion of the interstitial fibroblast population as a possible mechanism for the profibrogenic effects of fibrinogen.

Fibrinogen deficiency is associated with reduced interstitial cell proliferation in UUO

Proliferation of interstitial fibroblasts has been identified as a leading mechanism in progressive renal fibrosis.[19,20] In agreement with this, we found a high rate of proliferation in interstitial fibroblasts in UUO kidneys from both groups. However, staining for cell cycling marker Ki67 exhibited significantly lower rates of interstitial cell proliferation in $Fib^{-/-}$ kidneys when compared with $Fib^{+/-}$ kidneys after 7 and 14 days (Figure 4a). In contrast, no difference was observed in the number of proliferating tubular epithelial cells between the groups (Supplementary Figure S1C online). These findings suggest that fibrinogen exerts a profibrogenic effect by enhancing the proliferation of interstitial fibroblasts.

Fibrinogen directly mediates the proliferation of renal fibroblasts *in vitro*

It has been previously demonstrated that fibrinogen is a mitogen for some cell types[21] but not for others.[7] Using normal rat kidney interstitial fibroblasts (NRK-49F cells), we found that exposure to fibrinogen significantly increased cell proliferation in a dose-dependent manner (Figure 4b). This effect was cell-type specific, as no changes in proliferation were observed in renal epithelial cells (mPT and IMCD3 cells; Supplementary Figure S1D and E online). To substantiate the observed effect, we exposed renal fibroblasts to plasma from $Fib^{-/-}$ and $Fib^{+/-}$ mice. Consistent with the first results, we found a significantly stronger proliferation using fibrinogen-containing plasma when compared with fibrinogen-free plasma (Figure 4c). Collectively, these *in vitro* data indicate that fibrinogen acts as a direct mitogen on renal interstitial fibroblasts.

Fibrinogen does not promote the conversion of fibroblasts into myofibroblasts

The transition of resting fibroblasts into active α-SMA-positive myofibroblasts is regarded as a critical event in renal