Circulating fibronectin contributes to mesangial expansion in a murine model of type 1 diabetes

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Fibronectin is ubiquitously expressed in the extracellular matrix, and its accumulation in the glomerular mesangium in diabetic nephropathy is associated with deterioration of renal function in these patients. However, the exact role of fibronectin in the pathogenesis of diabetic nephropathy remains unknown. To clarify this, we administered fluorescent-labeled plasma fibronectin to wild-type mice and found it to accumulate in the mesangium. Using liver-specific conditional-knockout mice to decrease circulating fibronectin, we reduced circulating fibronectin by more than 90%. In streptozotocin-induced diabetes of these knockout mice, the pronounced fall in circulating fibronectin resulted in a decrease in mesangial expansion by 25% and a decline in albuminuria by 30% compared to diabetic control mice. Indeed, the amount of fibronectin in the kidney was reduced, as was the total amount of collagen. *In vitro* experiments confirmed that matrix accumulation of fibronectin was enhanced by increasing fibronectin only, glucose only, or the combination of both. Thus, circulating fibronectin contributes to mesangial expansion and exacerbation of albuminuria in a murine model of type 1 diabetes.

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Diabetic nephropathy is a major complication in patients with diabetes mellitus that results in the deterioration of renal function, the development of albuminuria, and eventually renal failure.[1] A characteristic pathologic finding in the kidney is the development of mesangial expansion, which reflects accumulation of extracellular matrix.[2,3] Because the local cellular environment in the glomerulus is considered the culprit responsible for extracellular matrix production, research has been focusing on modulating the intracellular signals in the various cells of the glomerulus, even though interest in the role of extracellular factors such as glycation of matrix proteins and matrix accumulation has been increasing.[4–7]

Fibronectin is one of the extracellular matrix proteins that accumulate in the expanded mesangial matrix in patients with diabetic nephropathy. It is produced by many cell types including mesangial cells.[8] Therefore, it has been assumed that the accumulated matrix originates in the cells in the mesangium.[9] It is involved in several vital cellular functions and modulates proliferation, survival, and differentiation of cells.[10–13] These various effects are mediated by the presence of several isoforms, such as the isoform containing the extra domain (ED) A (EDA) or EDB that affects a variety of cell surface receptors.[14] These isoforms are classically viewed as cellular, denoting a role in the immediate microenvironment of the cell producing these isoforms, but small amounts have also been detected in the circulation. Because fibronectin deletion in mice results in embryonal death, its role has been evaluated using conditional deletion in various organs. Over the past decade, a role for circulating fibronectin, which originates in the liver and lacks the 2 EDs, has been found in various contexts such as bone matrix, stroke, and cancer.[10,12,15] This is due to its ability to deposit in a variety of tissues and organs.[10,16,17] *In vitro* studies have shown that cells including mesangial cells require fibronectin to deposit collagen in their surrounding matrix, adding another layer of complexity in the study of this molecule.[18,19] Even though, to our knowledge, no studies exist on changes in total circulating fibronectin in diabetes, 1 study evaluated the amount of the EDA isoform in the circulation of diabetic patients and found it to be increased,[20] whereas another evaluated lectin-reactive fibronectin in the prediction of gestational diabetes.[21] Because EDA-containing fibronectin in the circulation is <5%, the relevance of these findings is not yet resolved.[16,22] The aim of

this work was therefore to determine whether circulating fibronectin plays any role in mesangial expansion in the presence of high plasma concentrations of glucose.

Using transgenic mice in which fibronectin was deleted in the circulation, we could show that circulating fibronectin contributed to mesangial expansion and albuminuria in diabetic nephropathy.

RESULTS

Systemically injected fibronectin deposits in renal glomeruli

Circulating fibronectin infiltrates various tissues.[10,16,17,23] To begin to address the role of circulating fibronectin in the kidney, we labeled purified plasma fibronectin with a fluorescent dye, injected it in wild-type mice i.p., and examined the kidneys histologically. Fluorescence was detected in the glomeruli but not in other parts of the kidneys (Figure 1a). Labeled albumin or the labeling dye injected alone, on the other hand, could not be detected in kidney sections (Supplementary Figure S1). In order to determine where labeled fibronectin localized in the glomeruli, we stained endothelial cells (von Willebrand factor),[24] podocytes (glial fibrillary acidic protein),[25] mesangial cells (desmin),[26] as well as the basement membrane and mesangial matrix (laminin).[27] As shown in Figure 1b, labeled fibronectin colocalized with laminin, but not desmin, and accumulated next to the desmin-stained mesangial cells. It did not stain endothelial cells or podocytes (Supplementary Figure S1). Because laminin is expressed in the basal membrane and the mesangial matrix,[28] we conclude that injected labeled fibronectin was incorporated within the mesangial matrix.

Deletion of circulating fibronectin diminishes fibronectin protein in the kidney

As fibronectin knockout mice die *in utero*, deletion of fibronectin in the circulation can be accomplished by using the albumin promoter (active in hepatocytes only, which represent the source of circulating fibronectin) attached to cre recombinase in mice homozygous for floxed fibronectin (conditional knockout [cKO]).[15,29] Deletion of fibronectin has been found to be complete by 8 weeks of age; this was confirmed by measuring circulating fibronectin compared with littermate control (CT) mice lacking cre recombinase expression (Figure 2a).

Loss of circulating fibronectin was associated with decreased fibronectin protein in the kidney as evidenced by staining, enzyme-linked immunosorbent assay (ELISA), and Western blot. Fibronectin mRNA was unchanged (Figure 2b–e). This cKO model thus allowed the study of the effect of loss of circulating fibronectin and the implications of decreased fibronectin incorporation in the matrix.

Deletion of circulating fibronectin diminishes mesangial expansion in a type 1 diabetes mellitus model in mice

Because injected labeled fibronectin was incorporated in areas normally affected by glomerular disease in diabetes mellitus (DM), we induced DM in CT and cKO mice. Elevated glucose levels were maintained for 24 weeks followed by killing. Evaluation of circulating fibronectin revealed a slight increase in diabetic cKO animals. The degree of deletion remained above 90%, however (Figure 3a). Survival was similar in diabetic cKO and CT mice and in healthy CT and cKO mice, but more diabetic mice died than healthy mice (Figure 3b,

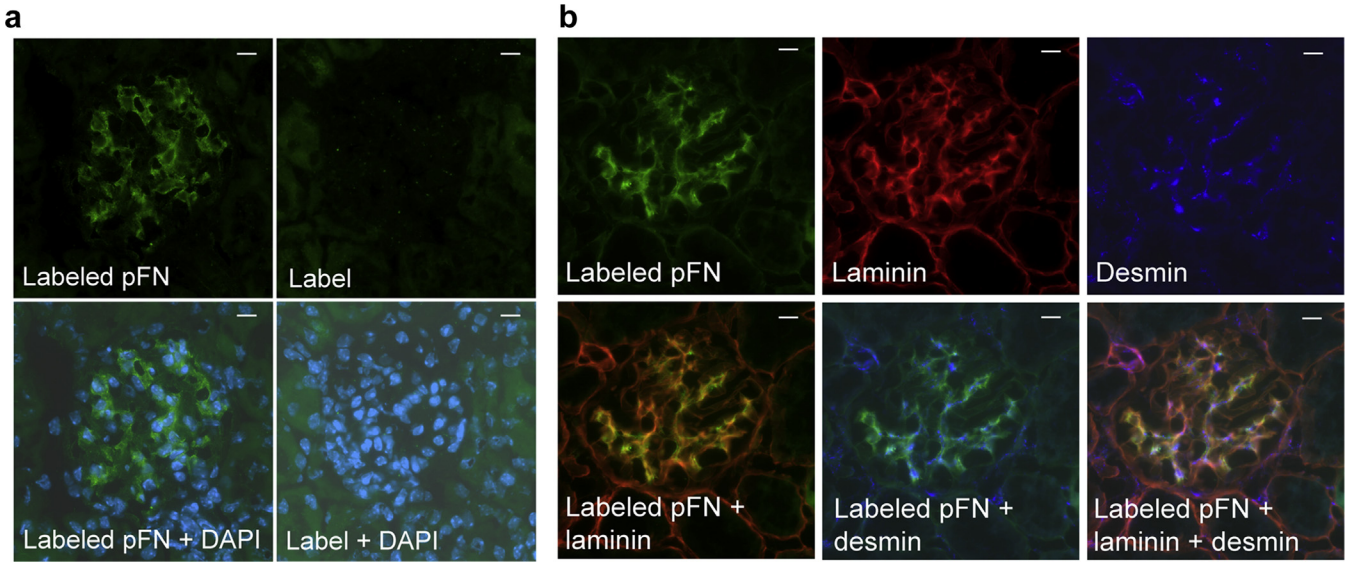

Figure 1 | Injected labeled fibronectin infiltrates the mesangium in healthy mice. (**a**) Labeled fibronectin (in green) is found in the glomeruli, whereas the label alone does not get incorporated in the glomerulus. Plasma fibronectin (pFN) was labeled with Oyster-500 and injected i.p. 3 times (days 1–3); as a control, the label alone was injected at the same time points. On day 5, mice were killed and kidneys were processed. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei (in blue); 4 mice per group. Bars = 10 μm. (**b**) Staining of laminin (in red) to detect the mesangial matrix and basement membrane and staining of desmin (in blue) to detect mesangial cells showed that fibronectin overlaps with laminin and the mesangium (shown in the overlays); number of mice as in (**a**). Bars = 10 μm.