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Collagen receptors integrin alpha2beta1 and discoidin domain receptor 1 regulate maturation of the glomerular basement membrane and loss of integrin alpha2beta1 delays kidney fibrosis in COL4A3 knockout mice

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ABSTRACT

Maturation of the glomerular basement membrane (GBM) is essential for maintaining the integrity of the renal filtration barrier. Impaired maturation causes proteinuria and renal fibrosis in the type IV collagen disease Alport syndrome. This study evaluates the role of collagen receptors in maturation of the GBM, matrix accumulation and renal fibrosis by using mice deficient for discoidin domain receptor 1 (*DDR1*), integrin subunit α2 (*ITGA2*), and type IV collagen α3 (*COL4A3*). Loss of both collagen receptors DDR1 and integrin α2β1 delays maturation of the GBM: due to a porous GBM filtration barrier high molecular weight proteinuria that more than doubles between day 60 and day 100. Thereafter, maturation of the GBM causes proteinuria to drop down to one tenth until day 200. Proteinuria and the porous GBM cause accumulation of glomerular and tubulointerstitial matrix, which both decrease significantly after GBM-maturation until day 250. In parallel, in a disease with impaired GBM-maturation such as Alport syndrome, loss of integrin α2β1 positively delays renal fibrosis: *COL4A3*$^{-/-}$/*ITGA2*$^{-/-}$ double knockouts exhibited reduced proteinuria and urea nitrogen compared to *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$ and *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ mice. The double knockouts lived 20% longer and showed less glomerular and tubulointerstitial extracellular matrix deposition than the *COL4A3*$^{-/-}$ Alport mice with normal integrin α2β1 expression. Electron microscopy illustrated improvements in the glomerular basement membrane structure. MMP2, MMP9, MMP12 and TIMP1 were expressed at significantly higher levels (compared to wild-type mice) in *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice, but not in *COL4A3*$^{+/+}$/*ITGA2*$^{-/-}$ mice. In conclusion, the collagen receptors DDR1 and integrin α2β1 contribute to regulate GBM-maturation and to control matrix accumulation. As demonstrated in the type IV collagen disease Alport syndrome, glomerular cell–matrix interactions via collagen receptors play an important role in the progression of renal fibrosis.

1. Introduction

The glomerular basement membrane (GBM) is responsible for the permselectivity and mechanical stability of the glomerular filtration unit (Kriz, 2008). Three different type IV collagen trimers are deposited in basement membranes: α1/α1/α2, α3/α4/α5, and α5/α5/α6 (IV) (Boutaud et al., 2000). The mature GBM predominantly contains α3/α4/α5 type IV collagen chains that are exclusively produced by podocytes (Abrahamson et al., 2009). Mutations in the type IV collagen genes COL4A3/4/5, which encode the α3/α4/α5 chains, cause Alport syndrome (AS). These mutations interfere with the correct assembly of the α3/α4/α5 (IV) collagen network in the GBM and hinder the developmental switch from the embryonic α1/α1/α2 (IV) network to the mature α3/α4/α5 (IV) network, causing the persistence of an immature GBM (Kalluri et al., 1997; Abrahamson et al., 2003). Thus, the GBM in Alport syndrome patients consists of α1/α2 (IV) chains only, making this altered GBM more porous and more susceptible to endoproteolysis (Kalluri et al., 1997). Consequently, a thickening and splitting of the GBM in AS causes progressive renal fibrosis leading to end-stage renal failure.

Several animal models of AS develop phenotypes that mimic human AS (Cosgrove et al., 1996; Lees et al., 1999). *COL4A3*$^{-/-}$ (Alport) mice exhibit progressive glomerulopathy that leads to a characteristic thickening of the GBM, an accumulation of extracellular matrix, proteinuria and progressive renal fibrosis (Cosgrove et al., 1996). Ultimately, Alport mice die from end-stage renal failure within weeks after birth.

Integrins are cellular transmembrane receptors for extracellular matrix components (Hynes, 2002; Barczyk et al., 2010). Specifically, the integrin-mediated contact between cells and the surrounding

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collagen plays an important role in renal development (Mathew et al., 2012). In Alport pathogenesis, it has been hypothesized that, due to an impaired recognition of defective type IV collagen in the GBM, extracellular matrix synthesis is deregulated via podocyte collagen receptors (Cosgrove et al., 2000; Gross et al., 2010). Cosgrove et al. (2000) analyzed the role of integrin α1β1 in Alport pathogenesis: *COL4A3*$^{-/-}$ Alport mice deficient in integrin α1β1 showed less expansion of mesangial matrix and less podocyte foot process effacement compared to *COL4A3*$^{-/-}$ mice with normal integrin α1β1 expression.

The expression of matrix metalloproteases (MMPs) MMP2, MMP9 and MMP14 was less pronounced than in *COL4A3*$^{-/-}$ Alport mice with normal integrin α1β1 expression (Cosgrove et al., 2008), resulting in reduced matrix deposition (or possibly caused by less matrix degradation). This finding underlines the importance of collagen receptors for the integrity of the basement membrane in the kidney. Similar to integrin α1β1, ablation of the DDR1 collagen receptor also improved the glomerular ultrastructure in Alport mice and significantly increased their lifespan by delaying end-stage renal failure (Cosgrove et al., 2008; Gross et al., 2010).

In general, the GBM structure is maintained by an equilibrium of synthesis and degradation. In Alport pathogenesis, increased synthesis of defective α3/α4/α5 type IV collagen, immature α1/α1/α2 type IV collagen and other basement membrane components in the GBM results in excessive accumulation of matrix proteins (Kruegel et al., 2013).

In the present report, we describe the impact of ablating the function of two collagen receptors, DDR1 and integrin α2β1, on the GBM-filtration barrier of the kidney. Further, we describe the impact of ablating the function of the collagen receptor integrin α2β1 on the pathogenesis of Alport syndrome.

2. Results

2.1. The loss of two collagen receptors integrin α2β1 and DDR1 delays maturation of the glomerular basement membrane causing proteinuria and matrix accumulation

Light microscopy and CT-scan did not show striking differences between kidneys of *DDR1*$^{-/-}$/*ITGA2*$^{-/-}$ mice and wild-type controls at days 100, 150 and 250 (Supplemental material 1). The loss of both collagen receptors, however, caused marked loss of high molecular weight (MW) proteins used as a marker for a porous, immature GBM (Table 1 and Fig. 1a). Loss of high MW-proteins more than doubled until day 100 and gradually decreased at day 190 to 8% compared to day 60 (Table 1, $p < 0.05$). Albuminuria increased by 39% at day 100 and decreased to 48% at day 190 compared to day 60 ($p < 0.05$). Loss of low MW-proteins as a marker for tubular malfunction increased by 113% at day 100 and decreased to 46% at day 190 compared to day 60 ($p < 0.05$). Immunostaining for extracellular matrix further explained the glomerular cause of proteinuria: the glomerular matrix score was significantly higher at day 100 than at days 150 and 250, indicating that maturation of the GBM hinders further loss of high molecular proteins (Fig. 1b). In parallel, tubulointerstitial matrix deposition increased until day 150. Tubulointerstitial matrix deposition decreased significantly at day 250, which was interpreted as a marker for improved function of tubular cells.

Table 1
Proteinuria in DDR1/ITGA2 double knockouts between 60 and 190 days of life.
Glomerular damage is indicated by loss of high molecular weight proteins (above 68 kDa, such as transferrin or immunoglobulin G). Presence of low molecular weight proteins (such as α1-microglobulin or β2-microglobulin) reflects tubular dysfunction.

Age in days	60	80	100	120	140	160	190
High molecular weight proteins	**100%**	123%	**206%**	85%	21%	19%	**8%**
Albumin	**100%**	107%	**139%**	102%	70%	63%	**48%**
Low molecular weight proteins	**100%**	128%	**213%**	143%	155%	140%	**46%**

2.2. The loss of two collagen receptors integrin α2β1 and DDR1 causes localized splitting and increased permeability of the glomerular basement membrane

In contrast to light microscopy, electron microscopy revealed localized GBM-thickening in *DDR1*$^{-/-}$/*ITGA2*$^{-/-}$ mice (Fig. 2) similar to those described previously in DDR1$^{-/-}$ and ITGA2$^{-/-}$ mice (Gross et al., 2010; Kruegel et al., 2013). These alterations were interpreted as ultrastructural hints for the impaired permeability for high MW-proteins as shown in Fig. 1a. However, additional studies are needed to further elucidate the glomerular cause for high molecular weight proteinuria.

2.3. The loss of integrin α2β1 reduces interstitial fibrosis and glomerulosclerosis in an Alport mouse model of impaired GBM maturation

The GBM-phenotype of *DDR1*$^{-/-}$/*ITGA2*$^{-/-}$ mice led to the hypothesis that not only DDR1 can positively influence the course of the type IV collagen disease Alport syndrome (Gross et al., 2010), but also integrin α2. In order to test this hypothesis, basement membrane deposition in the kidneys of *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice was compared to that in *COL4A3*$^{-/-}$/*ITGA2*$^{-/-}$ double knockouts. Light microscopy revealed only light staining of the glomerular and tubular basement membranes in wild-type animals (Fig. 3a). In contrast, the *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice showed prominent laminin staining (Fig. 3b). These morphological changes were less pronounced in the *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$ mice that carried only one *ITGA2* allele (Fig. 3c). *DDR1*$^{-/-}$/*ITGA2*$^{-/-}$ mice did not show any significant fibronectin staining at any time points (data not shown).

The glomerulosclerosis score (Fig. 3e) of the *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice was 4.24 ± 0.72. This score was decreased by 16.3% in *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$ (3.54 ± 0.99; $p = 0.063$ vs. Alport mice) and significantly decreased by 60% in the double knockouts (1.69 ± 0.83; $p < 0.001$ vs. Alport mice; $p < 0.001$ vs. *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$). In contrast, the *COL4A3*$^{+/+}$/*ITGA2*$^{-/-}$ mice with normal *COL4A3* expression showed scores similar to those of the wild-type controls (1.25 ± 0.56 and 1.08 ± 0.68).

COL4A3$^{+/+}$/*ITGA2*$^{-/-}$ mice with normal collagen α3(IV) expression had tubulointerstitial fibrosis scores similar to those of the healthy wild-type controls (1.03 ± 0.50 and 0.97 ± 0.62) (Fig. 3f), whereas the *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice showed severe fibrosis (3.99 ± 0.83). The tubulointerstitial fibrosis was significantly improved by inactivating one allele of ITGA2 in the *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$ mice (3.31 ± 1.06 at 150 days, a decrease of 17.0% compared with *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice; $p = 0.003$), and fibrosis was improved even further (by 61.4%) in the double knockouts (1.54 ± 0.74 at day 150; $p < 0.001$ vs. *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice; a decrease of 53.5% compared with *COL4A3*$^{-/-}$/*ITGA2*$^{+/-}$, $p < 0.001$).

2.4. The ablation of integrin α2β1 improves GBM ultrastructure in Alport mice

The *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice showed a characteristic thickening and splitting of the GBM (Fig. 4b); these changes were less severe in the *COL4A3*$^{-/-}$/*ITGA2*$^{-/-}$ double knockouts (Fig. 4c). As described previously (Girgert et al., 2010), the loss of integrin α2β1 in mice with normal *COL4A3* expression (*COL4A3*$^{+/+}$/*ITGA2*$^{-/-}$) leads to minor GBM matrix accumulation without fibrotic changes (Fig. 4d). Higher magnification imaging showed that the GBM in the *COL4A3*$^{-/-}$/*ITGA2*$^{+/+}$ Alport mice (inset in Fig. 4b), and to a lesser extent in the double knockouts (Fig. 4c), contained fibrillar collagen, indicating scar tissue formation that was not present in the *ITGA2* knockouts with normal *COL4A3* expression (inset in Fig. 4d).