Proteinuria Impairs Podocyte Regeneration by Sequestering Retinoic Acid

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ABSTRACT
In CKD, the risk of kidney failure and death depends on the severity of proteinuria, which correlates with the extent of podocyte loss and glomerular scarring. We investigated whether proteinuria contributes directly to progressive glomerulosclerosis through the suppression of podocyte regeneration and found that individual components of proteinuria exert distinct effects on renal progenitor survival and differentiation toward a podocyte lineage. In particular, albumin prevented podocyte differentiation from human renal progenitors in vitro by sequestering retinoic acid, thus impairing retinoic acid response element (RARE)-mediated transcription of podocyte-specific genes. In mice with Adriamycin nephropathy, a model of human FSGS, blocking endogenous retinoic acid synthesis increased proteinuria and exacerbated glomerulosclerosis. This effect was related to a reduction in podocyte number, as validated through genetic podocyte labeling in NPHS2.Cre;mT/mG transgenic mice. In RARE-lacZ transgenic mice, albuminuria reduced retinoic acid bioavailability and impaired RARE activation in renal progenitors, inhibiting their differentiation into podocytes. Treatment with retinoic acid restored RARE activity and induced the expression of podocyte markers in renal progenitors, decreasing proteinuria and increasing podocyte number, as demonstrated in serial biopsy specimens. These results suggest that albumin loss through the damaged filtration barrier impairs podocyte regeneration by sequestering retinoic acid and promotes the generation of FSGS lesions. Our findings may explain why reducing proteinuria delays CKD progression and provide a biologic rationale for the clinical use of pharmacologic modulators to induce regression of glomerular diseases.


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of podocytes, which are critical components of the glomerular filtration barrier, is key for progression to glomerulosclerosis.\textsuperscript{3–5} Different degrees of podocyte loss correlate with the degrees of proteinuria and, as an increasing proportion of glomeruli become involved, a measurable reduction of the clearance function of the kidney ensues.\textsuperscript{3–5} Proteinuria independently predicts progression and renal outcome in diabetic and non-diabetic disease.\textsuperscript{6} Lowering proteinuria retards renal disease progression\textsuperscript{5,6} and can induce regression of glomerulosclerosis, supported by experimental\textsuperscript{6–10} and clinical\textsuperscript{6,11,12} studies. For example, in animal models, reduction of proteinuria induced by treatment with angiotensin-converting enzyme (ACE) inhibitors is associated with increased number of podocytes and regression of glomerulosclerosis.\textsuperscript{13} Indeed, although proteinuria have limited capacity to divide, they can potentially get replaced by a population of renal progenitor cells (RPCs) localized within the Bowman capsule.\textsuperscript{14–17} In this study, we hypothesized that proteinuria may interfere with the generation of novel podocytes via direct or indirect impairment of regenerative mechanisms.

\section*{RESULTS}

\subsection*{Transferrin and IgG Overload Affects Human RPC Survival, while Albumin Overload Impairs RPC Differentiation into Podocytes}

To investigate the effects of protein overload, primary cultures of human RPCs were exposed to different concentrations of human serum albumin (HSA), transferrin, or IgG, which are the circulating proteins that are mostly lost when proteinuria occurs. Treatment with transferrin and IgG induced a reduction of \( ^{[3]H} \)-thymidine incorporation (Figure 1A), which was not observed with HSA treatment (Figure 1A). However, viability of HSA-treated and -untreated RPCs was similar but was reduced after treatment with transferrin and IgG, as shown with a combined flow cytometry analysis for propidium iodide and annexin V staining (Figure 1B). The effect of exposure to HSA, transferrin, or IgG on RPC differentiation into podocytes was then evaluated. In agreement with a previous study,\textsuperscript{13} culturing of human RPCs in the differentiative medium VRAD (Vitamin D3, retinoic-acid-supplemented DMEM-F12) resulted in novel expression of nephrin (NPHS1) mRNA and protein (Figure 1, C and D). This effect was abolished in the presence of HSA (Figure 1, C and D), but not in the presence of transferrin and IgG (Figure 1, C and D). HSA also blocked VRAD-induced acquisition of other podocyte-specific markers, such as podocin, podocalyxin (PODXL), and KLF15\textsuperscript{18} (Supplemental Figure 1A) and upregulation of other molecules necessary for podocyte function, such as p21, p27, and cyclin I (Supplemental Figure 1B). These results demonstrate that transferrin and IgG overload affects human RPC survival, while albumin overload impairs RPC differentiation into podocytes.

\subsection*{Albumin Overload Impairs Podocyte Differentiation by Sequestering Retinoic Acid}

We then analyzed the effects of distinct VRAD components on nephrin mRNA levels and demonstrated that retinoic acid (RA) exerted the strongest differentiative effect, while the effect of vitamin D3 was mild (Figure 2, A and B). HSA abolished RA-induced RPC differentiation into podocytes starting at 0.1 mg/ml (Figure 2, C and D). HSA can generate retinoid-HSA complexes through interaction with specific binding sites. We thus investigated the capacity of HSA to impair RA entry within RPCs by exposing them to \( ^{[3]H} \)-RA in the presence or absence of HSA and measuring radioactivity in supernatant and cells. In the absence of HSA, \( ^{[3]H} \)-RA could enter RPCs, whereas in the presence of HSA it remained in the supernatant (Figure 2E). Because RA can upregulate NPHS1 and other podocyte genes through activation of RA response elements (RARE) on their promoters, we infected RPCs with a RARE-reporter luciferase vector. As expected, HSA abolished the RA-induced activation of the RARE-reporter (Figure 2F). Taken together, these results demonstrate that HSA blocks the differentiation activity of 100 \( \mu \text{M} \) RA, which is contained in the VRAD medium. However, RA could induce RPC differentiation into podocytes starting already from a 10 \( \text{ng} \) RA dose (Figure 2G). In addition, RPCs displayed retinaldehyde dehydrogenases (Aldh1a1,a2,a3) mRNA expression (Supplemental Figure 2A) and Aldh1a enzymatic activity, as demonstrated by Aldefluor staining and sensitivity to diethylaminobenzaldehyde (DEAB) treatment (Figure 2H), thus showing that RPCs can synthesize RA from retinol. Consistently, retinol administration induced differentiation of RPCs into podocytes, an effect that was abolished by HSA (Figure 2I–L and Supplemental Figure 2B). This suggests that albumin can enter RPC and sequester endogenously synthesized RA. Consistently, after 1 hour exposure of RPCs to 1 mg/ml FITC-HSA, green fluorescence could be easily revealed within living cells (Figure 2M). In addition, pretreatment of RPCs with an inhibitor of protein kinase C, which is a mediator of albumin endocytosis, partially reverted the inhibitory effect of HSA on retinol-induced RPC differentiation into podocytes (54.5% \( \pm \) 5.3%; \( P<0.05 \)).\textsuperscript{19} Taken together these results demonstrate that HSA blocks RPC differentiation into podocytes induced by RA by preventing transcription of podocyte-specific genes.

\subsection*{In Vivo Models of FSGS to Evaluate the Effect of Albuminuria and RA on Podocyte Regeneration}

To evaluate the effect of albuminuria and RA on podocyte regeneration \textit{in vivo}, we used the Adriamycin nephropathy (AN) model, which mimics human FSGS and can be induced in different mice strains. We selected the three following backgrounds: (1) NPHS2.Crem1/mG transgenic mice, to validate markers that could be used in the other strains for analysis of podocyte loss and regeneration through podocyte genetic labeling. (2) Balb/c severe combined immunodeficiency (SCID) mice, to analyze the effects of endogenously synthesized RA in podocyte injury without confounding effects related to its
immunomodulatory activity; (3) RARE-lacZ transgenic mice, to visualize which cells are responding to RA. Evaluation of albumin, transferrin, and IgG levels in the urine through specific ELISAs after induction of AN demonstrated that in the experimental conditions established, all three mouse strains displayed similar levels of a nearly selective albuminuria (Figure 3A and Supplemental Figure 3). Evaluation of glomeruli in NPHS2.Cre;mT/mG transgenic mice demonstrated that proteinuria was related to a severe podocyte loss (Figure 3B). Indeed, in NPHS2.Cre;mT/mG transgenic mice, green fluorescent protein (GFP) is specifically and permanently expressed by podocytes independent of their functional status or pheno- typic appearance, making GFP loss a direct quantitation of podocyte loss. Three-dimensional reconstruction of glomeruli of NPHS2.Cre;mT/mG transgenic mice demonstrated severe podocyte depletion following induction of AN (Figure 3B). As a further confirmation, electron microscopy demonstrated podocytopenia in AN (Figure 3C). Interestingly, GFP expression coincided with that of Wilms tumor 1 (WT1) or nephrin in both healthy and AN mice (Figure 3, D and E); counting podocyte numbers as GFP-, or as WT1-, or as nephrin-expressing cells gave identical results (Figure 3F); GFP-expressing cells that had lost WT1 or nephrin were not observed (Figure 3G). Taken together, these results demonstrate that AN is characterized by a nearly selective albuminuria and that WT1 and nephrin provide an exact quantitation of podocyte numbers in this model.

Blocking Endogenous RA Synthesis Decreases Podocyte Number, Increases Proteinuria, and Enhances Mortality in SCID Mice with FSGS

To evaluate the in vivo effects of retinoids, we treated SCID mice affected by AN with different doses of Aldh1a inhibitor disulfram (DSF) or its vehicle. Treatment with 100 mg/kg and 200 mg/kg of DSF reduced survival in AN mice, while similar doses of DSF had no effect on healthy mice (Figure 4A). AN mice treated with DSF displayed a significantly higher albuminuria in comparison with vehicle-treated mice (Figure 4B). Indeed, at day 11, these mice displayed mean ± SD albuminuria values of 272.8±54.5 mg/dl versus 432.9±71.2 mg/dl in mice treated with DSF 100 mg/kg and 642.3±147.6 mg/dl in mice treated with DSF 200 mg/kg (P=0.05 and P=0.05). Albuminuria was absent in DSF-treated healthy mice, suggesting that blocking endogenous RA synthesis is not causing albuminuria per se (Figure 4B). Healthy mice consistently showed normal podocyte numbers, even after treatment with 100 mg/kg and 200 mg/kg of DSF (Supplemental Figure 4, A and B). In contrast, treatment with DSF in AN mice increased glomerulosclerosis (Figure 4C) by reducing podocyte number counted as WT1+claudin1− cells, representing differentiated podocytes that lost the progenitor marker claudin1 (Figure 4D, E, and F), or as nephrin+ cells (Supplemental Figure 4, A and B). These results demonstrate that endogenous synthesis of RA following podocyte injury limits albuminuria and glomerulosclerosis by increasing podocyte numbers.

RA Is Neutralized by Albuminuria after Podocyte Injury

To clarify the mechanisms of the RA-mediated effect, the RA synthetic pathway was assessed in RARE-lacZ transgenic AN mice. mRNA levels of key components of the RA biosynthetic pathway (RDH1,9,10, Aldh1a1,2,3) and the membrane

Figure 1. Transferrin and IgG overload affects human RPC survival, while albumin overload impairs RPC differentiation into podocytes. (A) Effect of HSA, transferrin, or IgG treatment on the proliferative capacity of RPCs, as assessed by [3H]-thymidine incorporation (n=4). (B) Flow cytometry analysis of RPC viability after treatment with HSA, transferrin, or IgG, as assessed by annexin-V and propidium iodide costaining. One representative of four experiments is shown (control: 90.82%; HSA: 90.35%; transferrin: 51.68%; IgG: 72.48%; P=0.05 control versus transferrin-treated and control versus IgG-treated cells by Mann-Whitney test, n=4). (C) NPHS1 mRNA levels in RPCs cultured in control medium or VRAD medium alone, or supplemented with HSA, transferrin, or IgG (n=6). (D) Nephrin protein expression assessed by FACS analysis in RPCs cultured in control medium or VRAD medium alone or supplemented with HSA, transferrin, or IgG. Representative histograms obtained in one of three independent experiments are shown. Percentages of nephrin-positive cells are calculated over their respective isotype control. All data are means ± SEM, **P<0.01 by ANOVA with Bonferroni post hoc analysis (A) and by Mann-Whitney test (C).
receptor for retinol-binding protein (STRA6) (Figure 5A), Aldh1a activity (Figure 5B), as well as expression of the Aldh1a enzymes (Figure 5C) were similar in glomeruli of AN mice and in normal glomeruli (Figure 5, A–C), suggesting similar levels of RA synthesis before and after injury. We then assessed RA and retinol levels in relation to levels of albuminuria in the

Figure 2. Albumin overload impairs RPC differentiation into podocyte by sequestering RA. (A) Effect of HSA (10 mg/ml) on NPHS1 mRNA levels in human cells cultured in control medium, VRAD medium, or RA- or vitamin D3 (vitD3)–containing medium (n=5). (B) Nephrin protein expression assessed by FACS analysis in RPCs cultured in control medium, VRAD medium, or RA- or vitamin D3–containing medium in presence or absence of HSA (10 mg/ml). Representative histograms obtained in one of three independent experiments are shown. Percentages of nephrin-positive cells are calculated over their respective isotype control. (C) Dose-response effect of HSA on NPHS1 mRNA levels in human RPCs cultured in RA-containing medium (n=4); P=0.0006 by ANOVA. (D) Nephrin protein expression assessed by FACS analysis in human RPCs cultured in control medium or in RA-containing medium, in presence of different doses of HSA (0.1, 1, or 10 mg/ml). Representative histograms obtained in one of three independent experiments are shown. Percentages of nephrin-positive cells are calculated over their respective isotype control. (E) Effect of increasing concentrations of HSA on the uptake of radiolabeled-RA by human RPCs (n=6); P<0.0001 by ANOVA for both curves. (F) Effect of HSA (10 mg/ml) on the transcriptional activity of RARE-reporter plasmid in infected human RPCs in control or RA-containing medium (n=6). (G) NPHS1 mRNA levels in human RPCs in response to increasing concentrations of RA (n=6). (H) Flow cytometry analysis of ALDH1a activity of human RPCs in presence (top) or absence (bottom) of the ALDH1a inhibitor DEAB. The flow cytometry gatings used are marked by boxes (ALDH1apositive fraction+DEAB: 0.67%±0.17% and–DEAB: 40.1%±1.83; n=4; P=0.02). (I) Effect of HSA (10 mg/ml) on NPHS1 mRNA induced by retinol treatment in human RPCs (n=6). (L) Nephrin protein expression assessed by FACS analysis in human RPCs cultured in control medium, retinol-containing medium, or retinol+HSA (10 mg/ml)–containing medium. Representative histograms obtained in one of three independent experiments are shown. Percentages of nephrin-positive cells are calculated over their respective isotype control. (M) Confocal microscopy analysis of FITC-HSA uptake by human RPC (green HSA signal, blue Topro-3 nuclear staining). One representative experiment of four is shown. Bars = 20 μm. All data are means ± SEM. *P<0.05, **P<0.01, ***P<0.001 by ANOVA with Bonferroni post hoc analysis for C, E, and G and by Mann-Whitney test for A, F, H, and I.
Figure 3. Evaluation of urinary protein levels and podocyte markers in mice with genetically tagged podocytes validate AN as a model to study podocyte loss and regeneration. (A) Evaluation of albumin, transferrin, and IgG levels in the urine of three different mice strains (n=15 each) after induction of AN (day 11). (B) Three-dimensional reconstruction of glomerulus of healthy and Adriamycin-treated NPHS2.Cre;mT/mG transgenic mouse (green = GFP). (C) Representative electron micrographs of glomerular podocytes in AN show
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progressively sequestered by albumin (Figure 5F). Decrease when albuminuria levels increase, suggesting that RA is allowing RA synthesis to occur (Figure 5F). However, urinary levels of RA increased proportionally to the severity of damage and allows RA synthesis to occur (Figure 5F). These results demonstrate that following injury, passage of retinol through the glomerular filtration barrier increases proportionally to the severity of damage and allows RA synthesis to occur (Figure 5F). However, urinary levels of RA do not increase in parallel with retinol levels but rather decrease when albuminuria levels increase, suggesting that RA is progressively sequestered by albumin (Figure 5F).

RA Treatment Increases Podocyte Number by Reversing Albuminuria-Impaired RARE Activation in RPC

We then aimed to counteract the harmful effect of albuminuria by administrating RA and evaluating which cells were responding to RA treatment in RARE-lacZ transgenic mice. Thus, 4 days after Adriamycin injection, mice were divided in two groups with identical proteinuria levels: one group was treated with RA and the other with vehicle. Mice treated with RA exhibited urinary doses of RA of 197±85.7 nM measured by LC-MS/MS. RA treatment (20 mg/kg) diminished proteinuria in RARE-lacZ transgenic mice, an effect that was significant after 1 week of treatment (Figure 6A). At day 11 after injury, vehicle-treated mice displayed high proteinuria, while proteinuria in RA-treated mice was significantly lower (774.4±214.7 mg/dl versus 170±50.2 mg/dl in RA-treated mice; P=0.04). There was no effect in healthy mice treated with RA or vehicle (Figure 6A).

Counting WT1+claudin1− or nephrin+ cells, we demonstrated that in RA-treated mice podocyte number is restored to a level slightly higher than the one observed at day 4 after Adriamycin treatment, although the difference was not statistically significant (Figure 6B and Supplemental Figure 6A). Thus, to establish whether RA was inducing generation of novel podocytes, avoiding the interindividual variability in podocyte numbers and in response to treatment, we performed serial renal biopsies in an additional group of 12 mice before and after RA treatment. Counting podocytes, we indeed demonstrated that RA increased WT1+claudin1− cells in 10 of 12 mice (Figure 6C). Similar results were obtained once podocytes were counted as nephrin+ cells (Supplemental Figure 6B). Interestingly, RA-treated mice also displayed more WT1+claudin1+ (RPCs that are differentiating into podocytes) compared with vehicle-treated mice, suggesting that RA was promoting RPC differentiation into podocytes.

While in healthy kidneys β-gal activity was not detected within glomeruli, but exclusively in scattered rare tubules (Figure 6D), 4 days after induction of podocyte injury, and just before starting RA or vehicle treatment, β-gal activity appeared in some RPCs lining the Bowman capsule or that were entering the glomerular tuft (Figure 6E). However, 7 days after RA treatment (i.e., 11 days after Adriamycin administration), β-gal activity was very different in RA-treated animals than in vehicle-treated mice (Figure 6, F and G). Indeed, at day 11, β-gal activity persisted and was further enhanced in RPCs of RA-treated mice (Figure 6F), which also started to acquire podocyte markers (Figure 6G), while β-gal activity was almost absent in vehicle-treated kidneys (Figure 6H). The results suggest that in mice treated with vehicle, endogenously produced RA was not available to induce RARE activation in RPCs. Indeed, RA-treated mice displayed more claudin1+β-gal+ RPCs compared with not only vehicle-treated mice (Figure 6I) but also mice analyzed immediately before initiation of treatment (Figure 6I). Among claudin1+β-gal+cells, 52.3%±6.4% also coexisted the podocyte marker nestin, and thus represented RPCs that were differentiating into podocytes. Interestingly, 16.02%±2.9% of RA-treated glomeruli showed at least one claudin1+β-gal+ cell, compared with only 1.7%±0.5% of glomeruli in vehicle-treated mice (P<0.0001) and 6.1%±0.77% of glomeruli in mice analyzed immediately before initiation of treatment (P=0.003). Although numerous claudin1+RPCs also localized within cellular FSGS lesions, almost all claudin1+ RPCs that exhibited β-gal activity localized outside these lesions, suggesting that RPCs within FSGS lesions cannot respond to RA and differentiate into podocytes (Figure 6I). Finally, 1 month after induction of injury, mice treated with RA exhibited improved renal function compared with vehicle-treated mice (Supplemental Figure 6C). Taken together, these results suggest that RA induces podocyte regeneration and that RA sequestration by proteinuria favors the generation of FSGS lesions.

DISCUSSION

Far from being a simple prognostic clinical measure, proteinuria has been proposed as a pivotal causative factor that

![Image of podocyte loss with extensive denudation of the capillary loops (arrows). Bars = 2 μm. (D) WT1 staining (blue) in renal sections of healthy (left) and of Adriamycin-treated (right) NPHS2.Cre;mt/mG transgenic mice (green = GFP; red = Tomato). Bars = 20 μm. (E) Nephrin staining (blue) in renal sections of healthy (left) and of Adriamycin-treated (right) NPHS2.Cre;mt/mG transgenic mice (green = GFP; red = Tomato). Bars = 20 μm. (F) Number of GFP, WT1, or nephrin-positive cells/glomerulus area in healthy (n=6) and in Adriamycin-treated (n=10) NPHS2.Cre;mt/mG transgenic mice. (G) Percentage of GFP cells coexpressing WT1 (left) or nephrin (right) in healthy (n=6) and in Adriamycin-treated (n=10) NPHS2.Cre;mt/mG transgenic mice. Data are means ± SEM; P=NS by Mann-Whitney test.]


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increases proteinuria and enhances mortality in SCID mice with FSGS. (A) Survival curve of Adriamycin-treated SCID mice exposed to DSF 100 mg/kg (purple line) (n=15) or 200 mg/kg (green line) (n=24). Survival curve of healthy mice (n=6), mice treated with DSF 100 mg/kg (n=6) and 200 mg/kg (n=6), and mice with Adriamycin alone (n=14) was 100%. (B) Time course assessment of albumin-to-creatinine ratio in mice with AN (blue triangle) (n=14) in comparison with mice with AN treated with DSF 100 mg/kg (pink inverted triangle) (n=11) or 200 mg/kg (green diamond) (n=11). Albumin-to-creatinine ratio in healthy mice and healthy mice treated with DSF 100 and 200 mg/kg (n=6 for each group) was all around 0. P=0.0001 by ANOVA test. (C) Representative periodic acid-Schiff staining of renal sections of healthy mice and of mice with AN, untreated or treated with DSF 100 and 200 mg/kg. (D) Representative WT1 (green) and claudin1 (red) co-staining of renal sections of healthy mice and of mice with AN, untreated or treated with DSF 100 and 200 mg/kg. (E) Left: index of glomerular injury assessed as described in the Concise Methods section. Right: quantitation of number of podocytes (WT1+claudin1– cells)/glomerulus is shown (healthy, n=6; Adriamycin, n=14; Adriamycin plus DSF 100 mg/kg, n=11; Adriamycin plus DSF 200 mg/kg, n=11). Topro-3 counterstains nuclei (blue). Bars = 20 μm. All data are means ± SEM. *P<0.05, **P<0.01 by Kaplan-Meier survival analysis and log-rank test for A, by ANOVA with Bonferroni post hoc analysis for B, and by Mann-Whitney test for E.

Figure 4. Blocking endogenous retinoic acid synthesis decreases podocyte number, increases proteinuria and enhances mortality in SCID mice with FSGS. (A) Survival curve of Adriamycin-treated SCID mice exposed to DSF 100 mg/kg (purple line) (n=15) or 200 mg/kg (green line) (n=24). Survival curve of healthy mice (n=6), mice treated with DSF 100 mg/kg (n=6) and 200 mg/kg (n=6), and mice with Adriamycin alone (n=14) was 100%. (B) Time course assessment of albumin-to-creatinine ratio in mice with AN (blue triangle) (n=14) in comparison with mice with AN treated with DSF 100 mg/kg (pink inverted triangle) (n=11) or 200 mg/kg (green diamond) (n=11). Albumin-to-creatinine ratio in healthy mice and healthy mice treated with DSF 100 and 200 mg/kg (n=6 for each group) was all around 0. P=0.0001 by ANOVA test. (C) Representative periodic acid-Schiff staining of renal sections of healthy mice and of mice with AN, untreated or treated with DSF 100 and 200 mg/kg. (D) Representative WT1 (green) and claudin1 (red) co-staining of renal sections of healthy mice and of mice with AN, untreated or treated with DSF 100 and 200 mg/kg. (E) Left: index of glomerular injury assessed as described in the Concise Methods section. Right: quantitation of number of podocytes (WT1+claudin1– cells)/glomerulus is shown (healthy, n=6; Adriamycin, n=14; Adriamycin plus DSF 100 mg/kg, n=11; Adriamycin plus DSF 200 mg/kg, n=11). Topro-3 counterstains nuclei (blue). Bars = 20 μm. All data are means ± SEM. *P<0.05, **P<0.01 by Kaplan-Meier survival analysis and log-rank test for A, by ANOVA with Bonferroni post hoc analysis for B, and by Mann-Whitney test for E.

 leads to a progressive increase of proteinuria, RPC response to RA is impaired and differentiation into podocytes is abolished. Taken together, these results provide a novel explanation of why albuminuria is a predictive factor of progression to glomerulosclerosis in patients with diabetic and nondiabetic nephropathies. Indeed, although podocytes can potentially be replaced by RPCs localized along the Bowman capsule, the regenerative capacity observed during glomerular disorders seems limited. Instead, aberrant RPC proliferation following podocyte injury may drive generation of hyperplastic glomerular lesions in patients with FSGS or collapsing glomerulopathy. Our results suggest that this occurs when RPC proliferate to replace injured podocytes, but they cannot fully differentiate into podocytes because of the presence of alleviation and therapy of podocyte number maintenance independent of its immunomodulatory effect.

Of note, by using genetic labeling of podocytes, we were able to demonstrate that in AN proteinuria was related to podocyte loss and not to dedifferentiation, indicating that RA modulates podocyte numbers. More important, using LC-MS/MS and confocal microscopy in RARE-lacZ transgenic mice, we provided evidence that following podocyte injury, retinol lost through the injured glomerular filtration barrier is transformed by Aldh1a into RA within the Bowman space. This activates RPC response to RA, thus initiating their differentiation into podocytes. However, once podocyte damage perpetuates kidney damage and precedes the relentless deterioration of renal function in progressive nephropathies. Previous studies showed that proteinuria has an intrinsic renal toxicity because of excessive reabsorption by proximal tubular cells, which causes activation of interstitial inflammation and fibrosis. However, why and how reduction of proteinuria is renoprotective for glomerular structures and preserves GFR is still unknown.

This study demonstrated that exposure to albumin can impair RPC differentiation into podocytes by sequestering RA, which is a critical driver of progenitor differentiation in multiorgan systems. Interestingly, RA was previously found to provide protection in multiple experimental models of kidney disease, including mesangioproliferative GN, puromycin-induced nephrosis, lupus nephritis, diabetic nephropathy, and HIV nephropathy. A recent study suggested that RA upregulates podocyte protein expression on parietal epithelial cells. In addition, treatment with RA reduced proteinuria in two patients with lupus nephritis. However, how RA improves kidney disease remains mostly unclear. Our data suggest that RA is essential for appropriate differentiation of RPCs into podocytes and that this process is altered by albumin in a dose-dependent manner. Albumin represents the specific carrier of RA in the blood and binds it with high affinity, which explains why even a small amount of albumin within the Bowman space can be detrimental for an appropriate regenerative process to proceed. In agreement with results of in vitro human RPC experiments, blocking endogenous RA synthesis in experimental FSGS induced in SCID mice markedly increased albuminuria and mortality, thus suggesting that endogenous RA synthesis is essential for podocyte number maintenance independent of its immunomodulatory effect. If note, by using genetic labeling of podocytes, we were able to demonstrate that in AN proteinuria was related to podocyte loss and not to dedifferentiation, indicating that RA modulates podocyte numbers. More important, using LC-MS/MS and confocal microscopy in RARE-lacZ transgenic mice, we provided evidence that following podocyte injury, retinol lost through the injured glomerular filtration barrier is transformed by Aldh1a into RA within the Bowman space. This activates RPC response to RA, thus initiating their differentiation into podocytes. However, once podocyte damage leads to a progressive increase of proteinuria, RPC response to RA is impaired and differentiation into podocytes is abolished. Taken together, these results provide a novel explanation of why albuminuria is a predictive factor of progression to glomerulosclerosis in patients with diabetic and nondiabetic nephropathies. Indeed, although podocytes can potentially be replaced by RPCs localized along the Bowman capsule, the regenerative capacity observed during glomerular disorders seems limited. Instead, aberrant RPC proliferation following podocyte injury may drive generation of hyperplastic glomerular lesions in patients with FSGS or collapsing glomerulopathy. Our results suggest that this occurs when RPC proliferate to replace injured podocytes, but they cannot fully differentiate into podocytes because of the presence of alleviation and therapy of podocyte number maintenance independent of its immunomodulatory effect.

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albuminuria. This leads to the amplification of an immature podocyte population, which is not able to interconnect with other podocytes within the glomerular tuft, thus generating glomerular hyperplastic cellular lesions. However, the regenerative process can possibly be rescued once the differentiative capacity of RPCs into podocytes is adequately restored, bypassing the neutralizing activity of albumin through exogenous administration of RA.

This is possible also because at the doses analyzed in this study, albuminuria does not appear to have a death-promoting effect in RPCs, in contrast to what was previously reported for podocytes. Indeed, progenitors usually exhibit an increased resistance to death compared with differentiated cells, which allows preservation of the possibility of starting a regenerative response following injury. Consistently, previous evidence in animals suggested that glomerulosclerosis can be reversed and the podocyte number increased once proteinuria is reduced. Similar results have also been reported in human studies. Indeed, findings from several clinical studies suggest that if proteinuria is reduced upon treatment with ACE inhibitors, recovery of kidney function due to regeneration is possible. Notably, a previous study reported that mouse treatment with ACE inhibitors induced regression of FSGS lesions and increased expression of podocyte markers by RPCs and podocyte number, suggesting that remodeling of RPCs is a key feature of ACE inhibitor renoprotection. Finally, our results also suggest that when both albuminuria and larger proteins, such as IgG and transferrin, are lost, a combined toxic effect not only blocks RPC differentiation into podocytes but also promotes their death. This potentially explains the observation that when nonselective proteinuria occurs, renal function declines faster.
Figure 6. RA treatment increases podocyte number by reversing proteinuria-impaired RARE activation in RPC. (A) Albumin-to-creatinine ratio in RARE-lacZ healthy transgenic mice, in healthy mice treated with RA, and in mice with AN with or without RA treatment. \( n = 32 \) at days 0 and 4, \( n = 11 \) for each group at the following time points. \( P = 0.0001 \) by ANOVA. (B) Quantitation of podocyte number (WT1+claudin1− cells)/glomerulus in healthy \( (n=10) \), Adriamycin before treatment \( (n=10) \), Adriamycin plus vehicle \( (n=11) \), Adriamycin plus RA \( (n=11) \). (C) Left: quantitation of podocyte number (WT1+claudin1− cells)/glomerulus in renal biopsy samples \( (n=12) \) before (bt) and after (at) RA treatment \( (3.98 \pm 0.6 \) versus \( 5.16 \pm 0.62) \). Center: quantitation of number of RPCs that are differentiating toward podocytes (WT1+claudin1+ cells)/glomerulus in renal biopsy specimens \( (n=12) \) before (bt) and after (at) RA treatment \( (2.80 \pm 0.31 \) versus \( 3.33 \pm 0.31) \). Each symbol represents one single mouse. Right: representative staining of WT1 (green) and claudin1 (red) in renal biopsy specimens of mice with AN before (top) and after (bottom) RA treatment. (D) Left: RARE activity (blue) in healthy mouse kidney. Right: higher magnification of colocalization of \( \beta\)-gal staining (blue) and claudin1 (red) assessed by immunohistochemistry (top) and immunofluorescence (bottom, \( \beta\)-gal green and claudin1 red). (E) Low and high magnification images showing RARE activity in the kidney of mice with AN at day 4 as seen by colocalization of X-gal staining (blue) and claudin1 (red) by immunohistochemistry (left) and by immunofluorescence (right) for \( \beta\)-gal (green) and claudin1 (red). Arrows point to positive glomeruli. (F) Low and high magnification
Taken together, these results support the concept that proteinuria represents not only a marker of progression but also a cause for glomerulosclerosis; they also explain why reducing proteinuria through proteinuria-lowering drugs delays progression of CKD. More important, the results of this study provide a biologic rationale for the clinical use of pharmacologic modulators to promote regression of glomerular disease.

**CONCISE METHODS**

**Human RPC Cultures**

Human RPCs were obtained and cultured as previously described, in agreement with the Ethics Committee on human experimentation of the Azienda Ospedaliero-Universitaria Careggi, Florence, Italy. Cell proliferation was assessed by [3H]-thymidine incorporation. HSA, the Azienda Ospedaliero-Universitaria Careggi, Florence, Italy. Cell agreement with the Ethics Committee on human experimentation of

9 ACGCCGGTGAAA CTCCCGCC-TAMRA-3

United Kingdom). For luciferase quantification, commercially available Assay on Demand kits (Applied Biosystems, Warrington, United Kingdom) to inhibit endocytosis.

With retinol was performed in the presence of 100 nM calphostin C

RA-induced differentiation was obtained by culturing the cells for 2 days with 10% FBS (Hyclone Laboratories, South Logan, UT), 100 nM vitamin D3, and 100 nM RA. In some experiments, differentiation with retinol was performed in the presence of 100 nM calphostin C (Tocris, Bristol, United Kingdom) to inhibit endocytosis.

**Propidium Iodide/Annexin V Staining**

Apoptosis and/or necrosis were evaluated using propidium iodide (Invitrogen, Carlsbad, CA) and the Annexin V kit (BD Biosciences, San Diego, CA) following manufacturer’s instructions, and the FACSDiva software.

**Real-Time Quantitative RT-PCR**

TaQMan RT-PCR was performed as described using commercially available Assay on Demand kits (Applied Biosystems, Warrington, United Kingdom). For luciferase quantification, the following primers and probes were used (Applied Biosystems): probe 5'-VIC-ACGCCGGTGAA ACTCCCGCC-TAMRA-3'; forward 5'-CGCA-GGTCTTCCCGACG-3'; reverse 5'-TTCCGTGCTCC AAAACAACA-3'.

**Immunofluorescence and Confocal Microscopy**

Confocal microscopy was performed by using an LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) as described. Primary antibodies (pAbs) were antinephrin pAb, antiretinaldehyde dehydrogenase 1/2 pAb (Aldh1a1/2, clone H-85) (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pan Cytokeratin mAb (PanCK, clone C-2562, Sigma), anti-β-galactosidase pAb (Abcam, Cambridge, United Kingdom), antiaclaudin1 pAb (Invitrogen), antiaclaudin1 pAb (Invitrogen), anti-WT1 mAb (clone F-6, Santa Cruz Biotechnology), antipodocin pAb (Alpha Diagnostics, MD), antinestin mAb (Santa Cruz Biotechnology). Secondary antibodies were AlexaFluor 488/647-labeled rabbit anti-goat IgG, AlexaFluor 488/546-labeled goat antirabbit IgG, AlexaFluor 488/546/633-labeled goat antimouse IgG1, AlexaFluor 633-labeled goat antirat (all from Molecular Probes, Invitrogen), AlexaFluor 488-labeled donkey antichicken (Jackson ImmunoResearch, West Grove, PA). Staining with FITC-conjugated HSA (Abcam) was performed following manufacturer’s instructions. Topro-3 (Molecular Probes) was used for nuclear staining.

**Flow Cytometry**

Intracytoplasmic staining for nephrin was performed as described elsewhere. A Goat IgG (Calbiochem, San Diego, CA, USA) was used as isotype control.

**RA Uptake Assay**

Cells were incubated in endothelial basal medium plus 5% FBS in the presence of 100 μM radiolabeled RA (all-trans-[11,12-3H(N)] RA, NEN Life Science Products, Boston, MA) and increasing concentrations of HSA (from 0 to 10 mg/ml). After 24 hours, supernatant and cells were collected and frozen, and radioactivity was determined using a β-counter (1205 Betaplate; Wallac/PerkinElmer, Waltham, MA).

**Human RPC Infection**

pGF1 (pGreenFire1)-RARE, pGF1-cytomegalovirus (CMV), and pGF1-mCMV (minimal CMV) plasmids were obtained from System Biosciences (Mountain View, CA). Lentiviral particles were produced by cotransfection of the lentiviral plasmid and the packaging vectors into Lenti-X 293T cells (Clontech, Mountain View, CA, USA). Cells were infected at a multiplicity of infection of 30, in the presence of 8 μg/ml of polybrene (Sigma). The pGF1-CMV plasmid was used as a positive control to estimate the infection efficiency by flow cytometry. Infection levels were further verified by quantitative PCR of luciferase DNA.

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For luciferase assay a commercially available kit (Promega, Milan, Italy) was used. The reporter activity of the pGFI-RARE-infected cells was measured on a GloMax 96 Luminometer (Promega) and normalized to the cells infected with pGFI-mCMV.

Evaluation of ALDH1a Activity

ALDH1a activity was assessed on human RPCs and mouse glomeruli using an Aldefluor staining kit (StemCell Technology, Vancouver, British Columbia, Canada) following the manufacturer’s recommendations.

Animals

Animal experiments were approved by the institutional review board; were performed in accordance with institutional, regional, and state guidelines; and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In Vivo Models of FSGS

AN was induced in three background strains by using doxorubicin (trade name Adriamycin). Because different mouse strains display variable sensitivity to Adriamycin treatment, different doses of the drug were administered to obtain damage of similar severity, as described below.

NPHS2.Cre;mT/mG Transgenic Mice

The NPHS2.Cre;mT/mG mice were developed to stably and irreversibly tag podocytes. Indeed, by crossing the transgenic NPHS2.Cre; mice with the mT/mG transgenic reporter strain, which carries a lacP-flanked Tomato cassette, Cre-mediated excision of membrane-targeted tandem dimer Tomato (mT) occurs, and an alternate reporter protein, membrane-targeted GFP, is selectively expressed in podocytes. Once recombination has occurred, GFP expression by the podocyte is not under control of the podocin promoter anymore but is continuously transcribed under control of enhancer elements from the chick b-actin gene and from CMV, thus allowing podocyte genetic labeling. B6.Cg-Tg (NPHS2-Tg)(295Sibf1) (here abbreviated NPHS2.Cre) and B6.129 (Cg)-Gr(ROSA)26Sor+tm4(ACTB-tdTomato,.EGFP)Luo/J (here abbreviated mT/mG) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). NPHS2.Cre;mT/mG reporter mice were generated by crossing mT/mG mice with NPHS2.Cre mice. To establish a model of Adriamycin-induced nephropathy in these mice, 6- to 10-week-old male mice were treated with Adriamycin by two successive retro-orbital injections on day 0.45% dehydrated in graded ethanol with a 2 hours in ice-cold 4% paraformaldehyde, then washed 30 minutes in ice-cold PBS and finally frozen; right kidneys were collected at the end of the experiment for analysis of renal morphology. Urinary albumin-to-creatinine ratio in 24-hour urine samples was evaluated on surviving mice on days 0, 3, 7, and 11 using Albuwel M kit (Exocell, Philadelphia, PA), and a creatinine assay kit (Cayman Chemical, Ann Arbor, MI). For the determination of urinary transferrin and IgG, a mouse transferrin ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) and mouse IgG ELISA kit (Cusabio, Wuhan, Hubei, P.R. China) were used, respectively. On day 11 after Adriamycin treatment, a 10.1%±0.5% decrease in body weight was observed. DSF treatment did not significantly affect body weight.

RARE-lacZ Transgenic Mice

RARE-lacZ transgenic mice (CD1 background) were purchased from the Jackson Laboratory. AN was induced in 6- to 10-week-old female mice by two successive retro-orbital injections of Adriamycin (14 mg/kg in PBS) on day 0 and 4 and on day 0 (n=32). Urinary albumin and creatinine in 24-hour urine specimens was evaluated on day 0, and on day 4, when mice were divided into two groups of 16 mice with similar proteinuria and 5 mice for each group were euthanized before starting treatment and kidneys collected for immunohistochemical analysis. Then, one group was subcutaneously injected with 20 mg/kg RA (stock 40 mg/ml in DMSO, diluted 1:4 in corn oil, n=11) and one with vehicle (DMSO, diluted 1:4 in corn oil, n=11) for 5 consecutive days. Proteinuria was evaluated again at days 8 and 11. BUN levels were measured by Reflotron System. Finally, on day 11 mice were euthanized and kidneys collected. Ten additional healthy mice were euthanized to evaluate control kidneys.

In an additional experiment AN was induced in 12 mice as described above. Animals were monitored for proteinuria and underwent biopsy on day 3 as follow: after anesthetization by intraperitoneal injection of 250 mg/kg 2,2,2-trichloroethanol (Avertin, Sigma), the kidney was exteriorized and a small piece of tissue was cut off using small scissors. A sterile gauze saturated with tranexamic acid (Tranex, Lusofarmaco, Peschiera Borromeo, Italy) was applied to the wound, which was then cauterized. The remaining kidney was pushed back in the body cavity and the animal sutured. From the day after the surgery, the animals were monitored for proteinuria and 5 mice for each group were euthanized before starting treatment and kidneys collected for immunohistochemical analysis. Then, one group was subcutaneously injected with 20 mg/kg RA (stock 40 mg/ml in DMSO, diluted 1:4 in corn oil, n=11) and one with vehicle (DMSO, diluted 1:4 in corn oil, n=11) for 5 consecutive days. Proteinuria was evaluated again at days 8 and 11. BUN levels were measured by Reflotron System. Finally, on day 11 mice were euthanized and kidneys collected. Ten additional healthy mice were euthanized to evaluate control kidneys.

SCID Mice

AN was induced in 6-week-old female SCID mice (Harlan, Udine, Italy) by a retro-orbital injection of Adriamycin (6 mg/kg in PBS; Sigma) on day 0 in a total of three independent experiments. Control mice received PBS. DSF (Sigma), 100 or 200 mg/kg in corn oil, or vehicle alone (corn oil) was given by oral gavage twice a week starting from 1 day before Adriamycin or PBS injection. Groups were defined as follows: (1) PBS plus vehicle (n=6); (2) PBS plus DSF 100 mg/kg (n=6); (3) PBS plus DSF 200 mg/kg (n=6); (4) Adriamycin plus vehicle (n=14); (5) Adriamycin plus DSF 100 mg/kg (n=15); (6) Adriamycin plus DSF 200 mg/kg (n=24). BUN levels were measured by Reflotron System (Roche Diagnostics, Rotkreuz, Switzerland). Kidneys were collected at the end of the experiment for analysis of renal morphology. Urinary albumin-to-creatinine ratio in 24-hour urine samples was evaluated on surviving mice on days 0, 3, 7, and 11 using Albuwel M kit (Exocell, Philadelphia, PA), and a creatinine assay kit (Cayman Chemical, Ann Arbor, MI). For the determination of urinary transferrin and IgG, a mouse transferrin ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) and mouse IgG ELISA kit (Cusabio, Wuhan, Hubei, P.R. China) were used, respectively. On day 11 after Adriamycin treatment, a 13.4%±0.7% decrease in body weight was observed. RA treatment did not significantly affect body weight.

Transmission Electron Microscopy

Kidneys were immersed in cold modified Karnovsky fixative containing 3% glutaraldehyde and 1% paraformaldehyde in PBS, post-fixed in phosphate-buffered 2% osmium tetroxide for 1 hour, dehydrated in graded ethanol with a final dehydration in propylene oxide, and embedded in Embed-812 (Electron Microscopy Sciences,
Ultrathin sections (approximately 90 nm thick) were stained with uranyl acetate and Venable lead citrate and viewed with a JEOL model 1200EX electron.

**Analysis of Glomerular Injury and Podocyte Count**

Kidney sections of 5 μm were fixed in ethanol and stained with periodic acid-Schiff reagent (Carlo Erba, Milan, Italy). Fifty randomly selected glomeruli were assessed for glomerular damage (well developed exudative, mesangial proliferation, and glomeruli hypertrophy) and graded as follows: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhesion involving <10% of the glomerulus; 2, sclerosis of 10%–20%; 3, sclerosis of 20%–30%; 4, sclerosis of 30%–40%; and 5, sclerosis >40% of the glomerulus. All scoring was performed in a blinded manner. For quantitation of podocytes, the number of nephrin-positive cells or of WT1+claudin1—cells or GFP-positive cells per glomerulus area was evaluated in 15 glomeruli of at least four sections for each mouse by two independent observers.

**LC-MS/MS**

One hundred microliters of mouse urine were treated by adding 1 μl of formic acid and then extracted twice with 0.5 ml of hexane. After phase separation, the hexane phase was dried under N2 stream and the residue dissolved in 100 μl of acetonitrile plus 0.1% of formic acid. Calibration curve was prepared by adding RA (stock solution 3 g/l in ethyl acetate) to urine to obtain concentrations of 0, 3, 30, and 300 μg/L and then treated as described above. The samples were immediately analyzed on an AB-Sciex QTRAP 5500 LC/MS/MS System (Applied Biosystems, Toronto, Ontario, Canada) equipped with an atmospheric pressure chemical ionization (APCI) source and coupled to a Series 1290 Infinity LC System (Agilent Technologies, Waldbronn, Germany) UHPLC Capillary Pump and the analytical column Kinex C18 2.6 μm, 3mm × 7/5 mm (Phenomenex, Torrance, CA). APCI source operated in positive ion mode, using the following setting: curtain gas 23, collision gas flow 9, temperature 400°C, nebulizer current 3. The following transitions were monitored for the multiple reaction monitoring experiment: m/z 301.1 > 205.3 (quantifier) and m/z 301.1 > 283.2 (qualifier). Optimal CE (collision energy), CXP (collision exit potential), and DP (declustering potential) were found at 17 and 50 V for both transitions.

Chromatographic separation was performed at room temperature at 0.4 ml/min flow rate using water (A) and acetonitrile (B), both containing 0.1% formic acid. The fast gradient started from a 100% of solution A. Chromatographic separation was performed at room temperature at 0.4 ml/min flow rate using water (A) and acetonitrile (B), both containing 0.1% formic acid. The fast gradient started from a 100% of solution A.


