Title: Podocyte as a direct target for treatment of glomerular disease?

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*Running title: Podocytes in glomerular disease

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The Centers for Disease Control and Prevention estimates more than 10% of adults in the United States, over 20 million Americans have chronic kidney disease (CKD). A failure to maintain the glomerular filtration barrier directly contributes to the onset of CKD. The visceral epithelial cells, podocytes, are integral to the maintenance of this renal filtration barrier. Direct podocyte injury contributes to the onset and progression of glomerular diseases such as Minimal Change Disease (MCD), Focal Segmental Glomerular Sclerosis (FSGS), Diabetic Nephropathy (DN), and HIV-associated nephropathy (HIVAN). Since podocytes are terminally differentiated with minimal capacity to self-replicate, they are extremely sensitive to cellular injury. In the past two decades, our understanding of the mechanism(s) by which podocyte injury occurs has greatly expanded. With this newfound knowledge, therapeutic strategies have shifted to identifying targets directed specifically at the podocyte. Although the systemic effects of these agents are important, their direct effect on the podocyte proves to be essential in ameliorating glomerular disease. In this review, we highlight the mechanisms by which these agents directly target the podocyte independent of its systemic effects.
INTRODUCTION

Chronic kidney disease (CKD) is a direct consequence of initial dysfunction and injury to the glomerular filtration barrier. The glomerular filtration barrier is composed of three critical components: visceral epithelial cells (podocytes), glomerular basement membrane, and endothelial cells. Although each of these three components is important to the maintenance of filtration barrier, injury to the podocyte remains central to the progression of primary glomerulopathies. Since podocytes are terminally differentiated epithelial cells with minimal capacity to regenerate, they are exquisitely sensitive to cell stress. Podocyte injury is integral to the pathogenesis of primary glomerulopathies such as Focal Segmental Glomerular Sclerosis (FSGS), Minimal Change Disease (MCD), and Membranous Nephropathy (MN). Although the inciting injury to the podocyte may vary between these glomerular diseases, the inevitable consequence of podocyte injury is actin cytoskeleton derangement, apical redistribution or loss of slit diaphragm proteins, and loss of structural integrity leading to eventual foot process effacement and podocyte detachment or apoptosis. Regardless of the type of insult, the podocyte ultimately loses its functional capacity to maintain the glomerular filtration barrier leading to progressive kidney disease. Consequently, therapies that prevent podocyte injury by enhancing their structural integrity, preventing their loss, or promote their regeneration will have a major clinical impact in our management of glomerular disease (Table 1). The focus of this review is to provide an overview of key therapeutic agents where the podocyte serves as their primary target of action in the treatment of primary glomerulopathies.

INHIBITING RENIN-ANGiotENSIN SYSTEM

In addition to the antihypertensive effects of renin-angiotensin system (RAS) inhibitors, angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II receptor blocker (ARB) play a critical role in mitigating podocyte injury and proteinuria in glomerular disease. Recent studies demonstrated that mechanical strain on podocytes results in upregulation of angiotensin II Type 1 receptor (AT1R) with eventual foot process effacement and proteinuria (9, 22). In addition, podocyte-specific overexpression of AT1R resulted in marked podocyte effacement and proteinuria without significant changes in systolic blood pressure (SBP) (9, 22). Consequently, the utilization of RAS inhibitors may have direct effects on the podocyte by inhibiting the effects mediated by AT1R. For instance, administration of aldosterone antagonists
such as spironolactone increased AKT phosphorylation leading to actin stabilization in the podocyte with reduction in proteinuria in the Ren2 rat model of hypertensive nephropathy (55). This improvement in podocyte injury and proteinuria were independent of changes in SBP (55). Furthermore, direct renin inhibitors such as aliskiren attenuated proteinuria and oxidative stress while improving nephrin expression in this Ren2 rat model, independent of AT1R blockade (24, 56). Interestingly, aliskiren-induced podocyte recovery was similar to that of irbesartan, despite not having as pronounced effect in SBP reduction (24, 56). Since (pro)renin receptor is present in human podocytes and activation of (pro)renin receptor triggers the generation of angiotensin peptides as well as MAPK/ERK signaling, blockade of the (pro)renin receptor directly attenuates proteinuria in murine models of diabetic and hypertensive nephropathy independent of changes in blood pressure (24). Interestingly, aliskiren reduced intracellular angiotensin II levels without inhibiting (pro)renin-induced MAPK/ERK signaling in cultured human podocytes, suggesting an independent pathway that can be targeted in RAS signaling (44). Nonetheless, the salutary benefits to the podocyte derived indirectly from the antihypertensive effects of inhibiting the RAS signaling cannot be neglected. For instance, the addition of AT1R antagonists attenuated glomerulosclerosis in 5/6 nephrectomy rat model with a concurrent reduction in SBP (1). Furthermore, administration of aldosterone increased oxidative stress and impairs endothelial nitric oxide synthase signaling, contributing to glomerular injury (1, 6). Taken together, inhibition of RAS signaling in the podocyte at multiple levels can attenuate podocyte injury and eventual loss by wide range of mechanisms, which include enhancing actin stabilization and reducing oxidative stress under models of podocytopathy.

**GLUCOCORTICOIDS**

Glucocorticoids (GCs) remain the initial and primary immunosuppressive therapy in the treatment of primary glomerulopathies (49). In many instances, alternate immunosuppressive therapy is typically not entertained until patients have failed GC therapy (46). Although the immunomodulatory effects of GCs are evident in pro-inflammatory states (4, 42), GCs exhibit a therapeutic benefit in primary glomerulopathies such as MCD and FSGS, where a pro-inflammatory milieu is not readily apparent. In fact, the initial treatment option for MCD is high dose GC therapy and, in many instances, alternate immunosuppressive therapy is typically not considered until patients have failed GC therapy (49).
GCs regulate gene expression by initially binding to the glucocorticoid receptors (GR) in the cytoplasm, which subsequently undergo dimerization and translocation to the nucleus and bind to glucocorticoid response elements on target genes. Previous studies have described the potential for GCs to have a direct effect on the podocyte by rearrangement of actin cytoskeleton, inhibiting apoptosis, and regulating protein trafficking of critical slit diaphragm proteins in murine and human podocytes (20, 38, 46, 51, 52, 57). In addition, GR as well as the major components of GR complex are expressed in human podocytes (20, 57, 58). Furthermore, GCs have also been implicated in ameliorating podocyte injury (38, 57) and improving podocyte survival (51) in murine and human cell culture models. Although, there is some evidence to suggest that GCs may have an anti-apoptotic effect by restoring Bcl-2 expression and reducing p53 levels in cultured podocytes treated with puromycin (51, 52), the mechanism mediating this process remains largely unexplored. In addition, others have demonstrated that GCs prevent podocyte cell death by downregulating cyclin kinase inhibitor p21 and IL-6 levels in cell culture models of podocyte injury (57).

Vascular endothelial growth factor (VEGF) is predominantly expressed in podocytes in normal glomeruli. However, in diabetic nephropathy, VEGF is markedly upregulated in podocytes and crosstalk with endothelial cells contributes to vasculogenesis and angiogenesis with subsequent vascular leakage and vasodilatation (3, 47, 57). In cultured human podocytes, treatment with GCs demonstrates a reduction in VEGF expression (57). However, others have demonstrated that targeting specific isoforms of VEGF is critical since complete inhibition of VEGF conversely leads to podocyte loss and proteinuria (11). In addition, VEGF-A and VEGF-C expression are essential for podocyte survival (35).

Endoplasmic reticulum (ER) stress can exacerbate podocyte injury (16). Specifically, ER stress leads to a failure in the synthesis of glycosylated nephrin, which is retained in the ER and fails to translocate to the podocyte membrane. By utilizing a cell culture model of energy depletion, the authors provide evidence that GCs reverse podocyte injury by restoring the synthesis of glycosylated nephrin by stimulating production of ATP (16).

Despite several groups demonstrating that the podocyte may serve as a direct target of GCs, a majority of the studies have been conducted in cell culture systems. A recent study by Zhang et al. demonstrated that administration of prednisone attenuated glomerulosclerosis in a proteinuric murine model of FSGS (60). The authors observed that prednisone reduced apoptosis
and increased podocyte progenitors by activating ERK signaling (60). However, additional studies are required to validate these findings and to identify whether key signaling molecules are required to mediate the effects of GCs in the podocyte. Combined, these studies demonstrate that the podocyte serves as a direct target of GCs, independent of its immunomodulatory effects.

**CALCINEURIN INHIBITORS**

Although GCs remain the initial treatment in patients with primary FSGS, only 25% of patients respond to initial therapy (8). In the GC-nonresponders, the next step in management is calcineurin inhibitors, where 40-50% of patients are expected to achieve partial or complete remission (17). Calcineurin is a serine/threonine phosphatase that dephosphorylates nuclear factor of activated T-cell (NFAT), which leads to nuclear translocation and activation of T cell-induced immune response (2). Although calcineurin inhibitors such as cyclosporine A and tacrolimus exhibit an immunosuppressive effect by inhibiting NFAT signaling in T cells, recent studies have determined that their therapeutic benefit may extend beyond immune cells.

Calcineurin is expressed in podocytes and calcineurin inhibitors have been demonstrated to ameliorate podocyte injury by restoring the expression of podocyte-specific proteins such as zonula occludens-1 (ZO-1) and synaptopodin (12, 25). Faul et al. determined that the beneficial effects of cyclosporin A in proteinuric murine models is directly related to stabilization of actin cytoskeleton in the podocyte under cell stress and independent of NFAT signaling in the podocyte (12). Specifically, the authors showed that cyclosporin A prevents synaptopodin from cathepsin-L mediated degradation leading to preservation of phosphorylated synaptopodin and subsequent RhoA-mediated stabilization of actin stress fibers under cell stress (12).

Since the podocyte membrane mediates the calcium influx required for activation of calcineurin, calcineurin inhibitors may also ameliorate podocyte injury by reducing the intracellular influx of calcium (26, 34, 45, 48). In addition to the effect on actin cytoskeleton, subsequent studies demonstrated that inhibition of NFAT signaling in the podocyte may also serve as a mechanism by which calcineurin inhibitors inhibit podocyte injury and eventual glomerulosclerosis (53).

Calcineurin inhibitors are associated with nephrotoxicity due to its pro-apoptotic effects in tubular and interstitial cells, which may potentially negate the therapeutic benefits in the podocyte. In cultured murine podocytes, Faul et al. demonstrated that cyclosporin A increased
actin stress fibers without inducing apoptosis. In contrast, other laboratories showed a dose-dependent increase in apoptosis with cyclosporin A treatment in cultured murine podocytes (14). Consequently, further studies are required to resolve these conflicting observations. Nonetheless, calcineurin inhibitors have a clear role in a subset of patients and should be included in the armament of therapeutic strategies in primary glomerulopathies.

**RITUXIMAB**

Several novel biological agents have emerged in recent years as potential therapies in patients with podocytopathies. Of these agents, rituximab has some promise in attenuating podocyte injury in patients with primary glomerulopathies who have failed GC or calcineurin inhibitor therapy. Rituximab is a chimeric monoclonal antibody directed at the CD20 glycoprotein on the surface of B cells. In addition to the immunosuppressive role of rituximab, recent studies demonstrate that the podocyte cytoskeleton is a direct target of Rituximab in patients with recurrent FSGS after renal transplantation (15). Specifically, Fornoni et al. determined that rituximab binds to the putative acid-sphingomyelinase, sphingomyelinase-like phosphodiesterase receptor (SMPDL-3b), on the podocyte membrane and prevents actin cytoskeleton derangement in recurrent FSGS (15). Despite these findings, rituximab has only been evaluated in small patient cohorts with primary FSGS. The response rate has only been 20-30%, suggesting that rituximab may only have a therapeutic benefit in a subset of patients with primary FSGS (13). Consequently, further studies are required to validate whether SMPDL-3b can identify the subset of patients that are likely to respond to rituximab therapy.

**ABATACEPT**

Co-stimulatory modulators such as B7-1 and B7-2 are expressed on the surface of antigen presenting cells and serve to regulate T-cell mediated immunity (43). Consequently, they have been primarily utilized for their immunomodulatory effects in kidney transplantation and other systemic anti-inflammatory disorders (7, 33). Interestingly, B7-1 is also expressed in podocytes and is significantly increased in models of podocyte injury (41). In addition, it has been demonstrated that urinary B7-1 levels correlate with the progression of MCD. Furthermore, Reiser et al. determined that knockdown of B7-1 prevented destabilization of the actin cytoskeleton in the podocyte and attenuated podocyte effacement and proteinuria in proteinuric
murine models (41). Most recently, Yu et al. demonstrated that abatacept, B7-1 inhibitor, restored β1 integrin levels, reversed podocyte migration, attenuated proteinuria, and reversed glomerular injury in a small cohort of patients with primary FSGS and recurrent FSGS after kidney transplantation (59). Although these findings need to be validated in larger cohorts, a subset of patients with B7-1 positive staining in primary FSGS may benefit from treatment with abatacept.

ACTH

Studies conducted more than a half century ago identified the potential therapeutic benefits of adrenocorticotropic hormone (ACTH) in pediatric cohorts with nephrotic syndrome (5, 39). ACTH is a pituitary neuroimmunoendocrine polypeptide that has been demonstrated to attenuate proteinuria in patients with nephrotic syndrome (19). Although its use in the treatment of podocytopathies had fallen off over the last century, it has recently emerged as a gel formulation in the United States. Although studies have identified the potential role of ACTH in patients with steroid-resistant FSGS, response rate remains less than 30% and its use as primary therapy has yet to be proven (23). Furthermore, the mechanism(s) by which ACTH attenuates podocyte injury remains unclear. Although some have postulated its direct role on the podocyte via the melanocortin 1 receptor (MC1R) in rat models of membranous nephropathy (28, 29), these findings were not observed in the proteinuric murine model of FSGS (29). However, Elvin et al. demonstrated that MC1R agonists only attenuated podocyte injury in cultured murine podocytes with overexpression of human MC1R (10). Future studies will need to focus on whether the conditional knockdown of the MC1R in podocytes attenuates the salutary effects of MC1R agonists. Detailed reviews have been published recently on the role of MC1R in podocyte injury and kidney disease (18, 19).

RETIONIC ACID

Retinoic acid (RA), derivative of vitamin A, is critical for cell differentiation, regulation of apoptosis, and inhibition of inflammation and proliferation. In the setting of podocyte injury, RA attenuates proteinuria and kidney injury in several animal models of proteinuric kidney disease (21, 27, 30-32). RA restores podocyte differentiation markers and may restore lost podocytes by inducing differentiation of kidney progenitor cells in proteinuric murine models. In
addition, treatment with RA attenuates inflammation and podocyte apoptosis in these proteinuric murine models.

Earlier studies demonstrate that treatment with RA reduced markers of proliferation and preserves podocyte-specific differentiation markers in models of HIV-associated nephropathy (HIVAN) (50). Since podocyte injury is due to a loss of podocyte differentiation markers in murine models of HIVAN, we determined that treatment with RA in cultured podocytes abrogates this process in a cyclic adenosine monophosphate (cAMP) dependent manner and via the induction of a kidney enriched zinc-finger transcription factor, Krüppel-Like Factor 15 (KLF15) (21, 32). Similarly the use of cAMP inhibitors diminished RA-induced podocyte differentiation (21, 27, 30). Furthermore, the use of phosphodiesterase-4 inhibitor increased cAMP levels and enhanced RA-induced differentiation markers in HIV-1 infected human podocytes (21). We also demonstrated that retinoic acid receptor alpha (RARα) is the key molecule mediating the reno-protective effects of RA and treatment with Am580, a RARα agonist, restores podocyte differentiation markers in proteinuric models of podocyte injury (40). In addition, the combined administration of phosphodiesterase inhibitor, Roflumilast, with Am580 resulted in a synergistic attenuation of proteinuria and podocyte injury in murine models of HIVAN (62). Combined, these findings suggest that treatment with RA restores podocyte differentiation markers via the RARα-mediated cAMP dependent pathway (21, 30).

RA has also been critical to the transition of parietal epithelial cells to podocytes in models of podocyte injury (37, 61). For instance, in a rat model of membranous nephropathy, RA restored podocyte number by increasing these epithelial transition cells in the glomerulus colocalized to Paired box gene 2 (PAX2), parietal epithelial cell marker, and Wilms-Tumor 1 (WT1), podocyte-specific marker (61). Similarly, the authors confirmed this using an experimental model of FSGS to illustrate that the beneficial effect of RA in podocyte regeneration (61). Since podocytes are terminally differentiated cells with minimal capacity to self-replicate, restoration of these podocytes by RA is critical to this regenerative process. However, this needs to be further confirmed by using the lineage tracing method. In addition, recent studies suggest that podocyte regeneration may not be sufficient to recover podocyte loss in CKD related to aging or nephron loss (54). Nonetheless, it would be interesting to determine whether podocyte regeneration induced by RA treatment has a significant contribution to its reno-protective effects.
Although RA has a therapeutic benefit in experimental models of glomerular disease, systemic toxicity has limited its use in the clinical setting. To list a few, the toxicity of RA includes hepatotoxicity, neurotoxicity, reduced spermiogenesis, and mucocutaneous side effects (36). For instance, the NIH sponsored open-label randomized clinical trial with the use of Isotretinoin in the treatment of patients with FSGS (NCT00098020) has been plagued with poor recruitment of subjects due to its toxicity. Recent derivatives of RARα agonist, Boronic Acid Retinoid (BD4), attenuate podocyte injury with less systemic toxicity in murine proteinuric models (63). Nonetheless, further studies are required to validate the efficacy of these novel derivatives in additional proteinuric murine models prior to studies in patients with primary glomerulopathies.

CONCLUSIONS

Our understanding of the pathophysiology and management of podocytopathies has dramatically changed over the last decade. In recent years, drugs repurposed to treat primary glomerulopathies demonstrate that their salutary benefits is in part due to their direct action on the podocyte. Based on these data, future studies must focus on identifying druggable downstream targets of these systemic agents that are specific to the podocyte with minimal systemic toxicity.

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DISCLOSURES

None
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• Reduce oxidative stress  
• Inhibit MAPK/ERK signaling |
| Glucocorticoids    | (16, 20, 38, 51, 52, 57, 58) | Murine and human podocytes, murine model (anti-glomerular antibody) | • Increase RhoA activity with stabilization of actin cytoskeleton  
• Reduces podocyte apoptosis  
• Restore Bcl-2 expression  
• Reduce p21, p53, Il-6, Vegf expression  
• Restores synthesis of glycosylated nephin |
| Calcineurin Inhibitors | (12, 45, 53) | Mouse model (LPS, transgenic mice), murine and human podocytes | • Restore ZO-1 expression  
• Prevents synaptopodin from cathepsin-L mediated degradation leading to preservation of phosphorylated synaptopodin and subsequent RhoA-mediated stabilization of actin stress fibers  
• Reduces calcium influx in the podocyte by downregulating TRPC6 expression  
• Inhibit NFAT signaling in the podocyte |
| Rituximab          | (15)       | Human podocytes, FSGS patient cohorts | • Rescues SMPDL-3b expression and prevents actin cytoskeleton derangement and podocyte apoptosis |
| Abatacept          | (41, 59)   | Mouse model (LPS), human podocytes, FSGS patient cohorts | • Inhibits B7-1 signaling and restores β1 integrin levels |
| ACTH               | (5, 28, 39) | Rat model (PhN), pediatric cohorts with nephrotic syndrome | • Binds to MC1R and attenuates oxidative stress |
| Retinoic Acid      | (21, 27, 30, 32, 37, 40, 50, 61-63) | Human podocytes, murine models (HIVAN, ADR, LPS) | • Binds to RARα and activates cAMP pathway  
• Induce KLF15 expression  
• Restores podocyte differentiation markers  
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