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Vitamin D Receptor Signaling in Renal and Cardiovascular Protection

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Abstract

The high prevalence of vitamin D-deficiency in patients with chronic kidney disease (CKD) is believed to be an important risk factor for the cardio-renal syndrome commonly seen in this patient population. African Americans suffer disproportionately high incidence of renal and cardiovascular disease with poor disease outcome, which may be partly attributed to their low vitamin D status in part due to low subcutaneous photo-production of vitamin D. Mounting evidence from animal and clinical studies has demonstrated beneficial effects of vitamin D therapy on the renal and cardiovascular systems, and the underlying reno- and cardio-protective mechanisms of vitamin D receptor (VDR)-mediated signaling are under intense investigations. In this article, I will review our most recent understanding of the renal protective mechanism of the podocyte VDR signaling against diabetic nephropathy and the anti-atherosclerotic role of macrophage VDR signaling in the regulation of atherosclerosis.

Keywords

Vitamin D-deficiency; renin-angiotensin system; podocyte; diabetic nephropathy; macrophage; atherosclerosis

Introduction

Vitamin D₃ is chemically converted from 7-dehydrocholesterol by ultraviolet light irradiation in the skin, and this is the main source for all vitamin D metabolites in the body. Thus sunlight exposure is required for vitamin D biosynthesis. Vitamin D₃ is hydroxylated to 25-hydroxyvitamin D (25(OH)D) by hepatic 25-hydroxylase, followed by further hydroxylation to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the most biologically active metabolite, by 25-hydroxyvitamin D 1-hydroxylase (CYP27b1) in the kidney. The pleiotropic biological activities of 1,25(OH)₂D₃ are mediated by the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily (1). The VDR is widely expressed in a variety of tissues/cells in the body, including cells not involved in calcium and phosphate metabolism, which is the basis for the broad biological functions of the VDR beyond the classic regulation of calcium and phosphorus homeostasis. As such, vitamin D-

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deficiency has detrimental impacts not only on the skeleton but also on extra-skeletal tissues, including the kidney and the cardiovascular system (2).

Traditionally vitamin D status is determined by circulating 25(OH)D levels in humans. Although still controversial, vitamin D-deficiency is usually defined as serum 25(OH)D 20 ng/mL or 50 nmol/L. As an emerging global health problem, vitamin D deficiency and insufficiency are believed to affect more than one billion people worldwide (3). The prevalence of vitamin D-deficiency is particularly high in patients with chronic kidney disease (CKD), mostly due to renal insufficiency. This includes decreased CYP27b1 activity for 1,25(OH)₂D₃ synthesis, impaired megalin-mediated endocytosis in the proximal tubular cells for reabsorption of 25(OH)D and an increase in FGF23 levels to suppress 1,25(OH)₂D₃ biosynthesis (4). Growing evidence suggests that vitamin D deficiency is a key risk factor for renal and cardiovascular problems commonly associated with CKD. In the African American population, because the high melanin content in the skin prevents UV light penetration leading to insufficient photo-production of vitamin D, vitamin D deficiency is very common. This genetically determined factor, on top of renal insufficiency, worsens vitamin D deficiency in African American patients with kidney disease. The severe degree of low vitamin D status is thought to contribute, at least in part, to the disproportionally high incidence of cardio-renal syndrome and poor disease outcome in African American patients (5). Recent investigations have greatly advanced our understanding of the protective and therapeutic mechanisms of vitamin D in renal and cardiovascular diseases (4). The focus of this article is to review our most recent research on the renal protective mechanism of the podocyte VDR signaling against diabetic nephropathy and on the role of macrophage VDR signaling in the inhibition of atherosclerosis.

Renoprotective Roles of Vitamin D-VDR Signaling

Mounting evidence reported in the past decade has demonstrated potent renoprotective activity of the vitamin D endocrine system. Numerous observational studies showed multiple beneficial effects of calcitriol or vitamin D analog therapy in both hemodialysis and non-dialysis CKD patients that led to significant survival advantage for the patients receiving the therapy (6–10). A key renoprotective function of vitamin D is to reduce albuminuria or proteinuria, major risk factors for CKD progression, renal failure, cardiovascular events and death (11). A cross-sectional analysis of the NHANES III database revealed a correlation between vitamin D-insufficiency and increased prevalence of albuminuria in the US adult population (12), suggesting that vitamin D has an intrinsic anti-proteinuric activity. Reduction of albuminuria or proteinuria is a major desired target for renoprotective therapy in CKD. Clinical studies have demonstrated therapeutic efficacy of vitamin D analogs to reduce proteinuria. Following a report that activated low calcemic vitamin D analog paricalcitol has significant anti-proteinuric activity in CKD (13), two small randomized pilot trials were conducted to evaluate the anti-proteinuric effect of oral paricalcitol treatment in CKD patients, and the results showed that paricalcitol significantly reduced albuminuria independent of its effect on hemodynamics or PTH suppression (14, 15). A following larger randomized placebo-controlled clinical trial confirmed that paricalcitol is able to reduce albuminuria and blood pressure in patients with diabetic nephropathy who were already on renin-angiotensin inhibitor therapy (16). These studies provide clinical evidence that low-calcemic vitamin D analogs can be used as an alternative or complementary therapy for treatment of proteinuria in CKD.

Studies with different experimental models of kidney disease have provided insights into the reno-protective mechanism for vitamin D and its analogs. These investigations show that vitamin D or its analogs protect the kidney by regulating multiple pathways known to play a role in kidney injury, including the renin-angiotensin system (RAS), NF- κ B, Wnt/ β -catenin and slit diaphragm proteins (4). We have shown that in diabetic state or under

unilateral ureteral obstruction (UUO) genetic deletion of VDR in mice leads to severe renal injury largely due to increased activation of the local RAS in the kidney, resulting in early onset and robust albuminuria, glomerulosclerosis and interstitial fibrosis (17, 18). Treatment with 1,25(OH)₂D₃ or activated vitamin D analogs reduced albuminuria and prevented podocyte injury in 5/6 nephrectomized rats (19–21), puromycin aminonucleoside-induced podocyte apoptosis (22), and adriamycin-induced nephropathy (23). We reported that vitamin D analog monotherapy or its combination therapy with inhibitors of the RAS reduced albuminuria and prevented podocyte loss in experimental models of type 1 and type 2 diabetes (24–26). Our research also showed that podocytes express the VDR that is highly inducible by 1,25(OH)₂D₃ (27), and through VDR, 1,25(OH)₂D₃ transcriptionally stimulates the expression of nephrin, a key slit diaphragm protein synthesized by podocytes (28). Thus, the vitamin D-VDR signaling plays a critical role to maintain the integrity of the slit diaphragm. Together recent research data suggest that podocytes may be a key antiproteinuric target of vitamin D (29), and our recent transgenic studies have demonstrated critical renoprotective activity of the podocyte VDR signaling.

Podocyte VDR Signaling against Diabetic Renal Injury

Podocytes play a key role in the regulation of glomerular filtration in the kidney. The foot processes of podocytes are an integral part of the glomerular filtration barrier that keeps proteins and other large molecules from being filtered into the urine. Podocytes synthesize proteins that are key components of the slit diaphragm formed between adjacent interdigitating foot processes that functions as the major size- and charge-selective barrier to protein leakage (30). Therefore, podocyte injury, loss or death leads to albuminuria (11). Recently we employed a transgenic (Tg) approach to explore the role of the podocyte VDR signaling in renoprotection using the streptozotocin (STZ)-induced diabetic nephropathy model.

We used the 2.5-kb human podocin gene (NEPH2) promoter to target Flag-tagged human (h) VDR to podocytes in DBA/2J mice, a genetic background known to be susceptible to diabetic renal injury (24, 26, 31). This podocin gene promoter has been well documented for its podocyte specificity in driving transgene expression (32). To test whether an increase in podocyte VDR would enhance the renoprotective effect of vitamin D, we compared the therapeutic efficacy of a low dose vitamin D analog doxercalciferol (Dox) between STZ-induced diabetic wild-type (WT) and hVDR-Tg mice. This dose of Dox (30 ng/kg, i.p. injection 3x per week) was 1/10th of the dose used previously to prevent albuminuria in diabetic WT mice (26). Although this low Dox dose had little effects on the development of albuminuria in WT mice, it almost completely blocked albuminuria in Tg mice (Fig. 1A). Thus podocyte VDR overexpression renders the mice more sensitive to vitamin D therapy. In fact, Tg mice exhibited significantly reduced albuminuria compared to WT mice even at baseline (Fig. 1A).

Further examination revealed that the hVDR transgene was able to mediate the activity of the low dose Dox to prevent hyperglycemia-induced kidney injury. These include prevention of glomerular VDR reduction (Fig. 1B), attenuation of podocyte loss (Fig. 1C and 1D), blockade of glomerular fibrosis (Fig. 1E and 1F) and glomerular fibronectin induction, a key extracellular matrix protein required for glomerulosclerosis (Fig. 1G), and reversing the decline of slit diaphragm proteins podocin and nephrin (Fig. 1H). The thickening of the glomerular basement membrane, a marker of glomerular filtration barrier dysfunction, was also prevented, and the morphology of podocyte foot processes preserved by the hVDR transgene. These protective events were not observed in low Doxtreated WT mice.

Mechanically we found that podocyte hVDR overexpression inhibited podocyte apoptosis induced by diabetes, demonstrated by TUNEL staining (Fig. 2A and 2B). We were also able to confirm in podocyte cultures that high glucose (30 mM) exposure induced podocyte apoptosis, manifested by increased nucleic condensation (Fig. 2C and 2D), DNA fragmentation (Fig. 2E) and caspase 3 activation (Fig. 2F), and these changes were markedly attenuated in the presence of 1,25(OH)₂D₃ treatment (Fig. 2C – F). In fact, high glucose-induced podocyte nuclear condensation could be blocked by p38 MAPK inhibitor SB203580 (Fig. 2G), and angiotensin (Ang II)-induced podocyte nuclear condensation could be blocked by AT1 receptor blocker losartan, as well as by SB203580 and 1,25(OH)₂D₃ (Fig. 2G). These observations suggest that 1,25(OH)₂D₃ inhibits podocyte apoptosis by targeting p38 activation.

The Bcl2 family members play important roles in apoptosis. We found that glomerular Bcl2 (anti-apoptotic) was moderately reduced, and Bad (pro-apoptotic) levels increased, in diabetic WT mice, which were reversed in the hVDR-Tg mice. In podocyte cultures, high glucose had little effects on p53, but induced Bad and Bak (pro-apoptotic), and these changes were diminished in the presence of 1,25(OH)₂D₃ pre-treatment. 1,25(OH)₂D₃ also increased Bcl2 levels, consistent with the notion that 1,25(OH)₂D₃-VDR signaling inhibits podocyte apoptosis. We further examined several intracellular signaling pathways known to promote apoptosis. Whereas high glucose and 1,25(OH)₂D₃ had little effects on Akt phosphorylation, high glucose quickly induced ERK phosphorylation, which was inhibited when the cells were pretreated with 1,25(OH)₂D₃. High glucose moderately induced p38MAPK phosphorylation, and this was not affected by 1,25(OH)₂D₃; however, like p38 inhibitor SB203580, 1,25(OH)₂D₃ inhibited high glucose-induced p38 activity to phosphorylate mitogenactivated protein kinase-activated protein kinase 2 (MAPKAPK2), a downstream substrate p38 kinase (33). Moreover, while Ang II is known to activate p38 to induce apoptosis (34), 1,25(OH)₂D₃ was also able to block Ang II-induced MAPKAPK2 phosphorylation. These data indicate that 1,25(OH)₂D₃ inhibits p38 activity and p38-mediated signaling in podocyte apoptosis.

We also examined the intrarenal RAS, a cascade known to play a key role in diabetic renal injury (35). Renin is the rate-limiting enzyme of this cascade, and Ang II is the effector of the RAS that is known to promote podocyte apoptosis *in vitro* and *in vivo* (36, 37). We observed glomerular renin up-regulation in diabetic WT mice, but in Tg mice, particularly in the presence of low Dox treatment, the increase in renin expression was blocked (Fig. 3A and 3B). Furthermore, we showed that in podocyte cultures high glucose induced the expression of renin and angiotensinogen (Fig. 3C), resulting in marked increases in intracellular renin activity and Ang II release in the media, and these inductions were blocked by 1,25(OH)₂D₃ (Fig. 3D and 3E). High glucose-induced AT1 receptor expression in podocytes was also attenuated by 1,25(OH)₂D₃. These data suggest that the 1,25(OH)₂D₃-VDR signaling inhibits the RAS activation in podocytes to suppress high glucose-induced podocyte apoptosis.

Reconstitution of Podocytes with hVDR

VDR-null (VDRKO) mice developed much more severe albuminuria and renal damage than WT mice in diabetes (17). To confirm the renoprotective role of podocyte VDR signaling, we asked whether the podocyte-specific hVDR transgene was able to rescue VDRKO mice from developing severe diabetic renal injury. To this end we reconstituted the podocytes of VDRKO mice with the hVDR transgene through crossing of Tg and VDRKO mice in DBA/2J background, resulting in transgenic VDRKO (Tg-KO) mice. In these Tg-KO mice only podocytes express hVDR and other cells are VDR negative (Fig. 4A). In STZ diabetes model VDRKO mice developed albuminuria more severe than WT mice as reported (17), but the severity was markedly attenuated in Tg-KO mice (Fig. 4B). Diabetes-induced

podocyte loss (Fig. 4C and 4D) and glomerular sclerosis (Fig. 4E and 4F) were also more severe in VDRKO mice compared to WT mice, and these phenotypes were ameliorated in Tg-KO mice (Fig. 4C – F). Electron microscopic examination of the glomerular filtration barrier revealed severe effacement of podocyte foot processes and thickened glomerular basement membrane in VDRKO mice, and these abnormalities were attenuated in Tg-KO mice (Fig. 4G and 4H). The dramatic increase in glomerular FN levels and decrease in nephrin levels seen in VDRKO mice were mostly reversed in Tg-KO mice (Fig. 4I and 4J). Reconstitution of the hVDR transgene in podocytes was also able to attenuate renin induction in the glomerulus (Fig. 4K). In the VDR-null background, the systemic level of $1,25(\text{OH})_2\text{D}_3$ is extremely high (>10 fold the normal level) because of the lack of feedback suppression (38, 39). In Tg-KO mice this high level of $1,25(\text{OH})_2\text{D}_3$ was not able to act on cells except the podocytes. Therefore, the rescue of diabetic renal injury observed in the Tg-KO mice is a very compelling piece of evidence that supports the importance of the podocyte VDR signaling in renoprotection. Taken together, these data demonstrate podocytes as a key therapeutic target in vitamin D therapy of chronic kidney disease.

Renoprotective Mechanisms of Podocyte VDR Signaling

These recent investigations in transgenic mice and podocyte cultures provide strong evidence that the VDR signaling in podocytes has potent renoprotective activities against diabetic nephropathy, and the podocyte VDR at least in part mediates the anti-proteinuric action of $1,25(\text{OH})_2\text{D}_3$ and its analogs. The central mechanistic basis of this protection is the inhibition of hyperglycemia-induced podocyte apoptosis, thought to be one major cause for podocyte loss in diabetic nephropathy (40). As podocytes express all components of the RAS that can be activated by high glucose (41, 42), and the pro-apoptotic activity of Ang II towards podocytes is well known (37), the activation of the local RAS in podocytes is able to induce apoptosis by an autocrine or paracrine fashion. We demonstrated that in Tg and Tg-KO mice the induction of renin in the glomeruli by hyperglycemia was attenuated, and in podocyte cultures high glucose-activated local RAS and Ang II was reversed by $1,25(\text{OH})_2\text{D}_3$. Therefore, it is clear that vitamin D protects podocytes through blockade of the local RAS activation (Fig. 5). Our data also demonstrated that vitamin D blocks high glucose-induced caspase 3 activation and pro-apoptotic pathway. High glucose induced activation of p38 and ERK in podocytes and mesangial cells has been documented to cause renal injury (43, 44), and $1,25(\text{OH})_2\text{D}_3$ abrogated ERK phosphorylation and inhibited p38 activity. This regulation may be direct or indirect via Ang II, as Ang II is known to activate ERK and p38 signaling pathways. We therefore speculate that vitamin D inhibits podocyte apoptosis by blocking these signaling pathways (Fig. 5). The induction of nephrin by $1,25(\text{OH})_2\text{D}_3$, as described previously (28), also contributes to the protection of podocytes (Fig. 5). Moreover, vitamin D analogs can also block Wnt/ β -catenin signaling in podocytes to ameliorate podocyte damage (23). Therefore podocyte VDR signaling likely protects the podocytes by targeting multiple pathways.

Association of Vitamin D Deficiency with Cardiovascular Disease

Ultraviolet irradiation is required for the cutaneous synthesis of vitamin D and for the maintenance of vitamin D status. Ultraviolet irradiation decreases with the increase in latitude, and high latitudes are correlated with high prevalence of hypertension and stroke incidents (45, 46). Dark skin pigmentation in the African American population, which blocks ultraviolet light penetration, is associated with higher blood pressure (47, 48). NHANES III data show an inverse relationship between serum $25(\text{OH})\text{D}$ and blood pressure in the general population (49). These data also reveal that serum $25(\text{OH})\text{D}$ levels are inversely associated with increased prevalence of cardiovascular risk factors including hypertension, diabetes, obesity and hyperlipidemia (50). Low vitamin D status is thought to be a contributing factor for congestive heart failure (51). Other epidemiological studies

confirm the association between vitamin D-deficiency and increased risk of cardiovascular problems, including myocardial infarction (52, 53). Low serum 25(OH)D is also associated with incident cardiovascular disease in Framingham Offspring Study participants without prior cardiovascular disease, after adjustment for C-reactive protein, physical activity or vitamin use (54). Our recent studies demonstrate that VDR signaling plays an important role in the inhibition of atherosclerosis.

Critical Roles of Macrophages in Atherosclerosis

Development of atherosclerotic lesions involves the interactions of modified lipoproteins, endothelial cells, macrophages, T cells and vascular smooth muscle cells in the arterial wall (55, 56). Atherosclerosis is usually initiated by elevated plasma lipids such as low-density lipoprotein (LDL), which infiltrates into the vascular wall and, after being modified, promotes local inflammation and macrophage lipid loading. This chain of events ultimately leads to complex vascular lesions. Modified LDL particles have multiple activities that promote atherosclerosis (55), including inducing adhesion molecules (57–59) and promoting monocyte infiltration (60, 61) and foam cell formation (62, 63).

Macrophages play an essential role in atherogenesis (62–64). Macrophages internalize modified lipoproteins (e.g. oxidized LDL) in an unregulated fashion, leading to formation of foam cells (65). Macrophages and foam cells induce adhesion molecules in endothelial cells and secrete chemokines and cytokines to promote monocyte recruitment and differentiation. As antigen-presenting cells, macrophages process peptides and lipids from the modified lipoproteins to present to T lymphocytes, and activated T cells augment the inflammatory response mediated by secreted cytokines, further driving atherosclerotic lesion progression (56). Macrophages also produce metalloproteinase and PAI-1, which can destabilize atherosclerotic plaques (55, 66), leading to plaque rupture. Macrophages express all components of the RAS, a potent promoter of atherosclerosis (67, 68). Ang II production is increased during monocyte to macrophage differentiation (69, 70). Specific deletion of renin in macrophages leads to significant reduction in atherosclerosis without altering circulating renin concentrations, which indicates the importance of the macrophage RAS in atherosclerosis (71).

Anti-atherosclerotic Activity of VDR Signaling

There is overwhelming epidemiological and clinical evidence that links vitamin D deficiency to cardiovascular disease (72), including coronary stenoses, coronary artery calcification and carotid atherosclerosis (73–75); however, our understanding of vitamin D in vascular disease remains very limited. Recent studies suggested that vitamin D is able to inhibit foam cell formation by suppressing cholesterol uptake in macrophages from type 2 diabetes patients (76) and to decrease atherosclerosis by regulating T lymphocyte functions (77). Our recent studies demonstrated that the VDR signaling inhibits atherosclerosis in part by blocking the activation of the local RAS in macrophages.

In these studies we used the LDL receptor (LDLR) knockout atherosclerosis model, and our experimental strategy was to compare the atherosclerotic phenotypes of LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice fed an atherogenic diet that contains high fat and high cholesterol (HFHC). Atherosclerotic lesions were assessed at four vascular sites: innominate artery (IA), ascending aorta (AA), descending aorta (DA), and the aortic root (AR) of the heart (78). The HFHC diet induces dramatic hyperlipidemia, but for reasons that are not fully understood, plasma cholesterol levels, including VLDL, LDL and HDL cholesterol, were significantly lower (by >30%) in LDLR^{-/-}/VDR^{-/-} mice compared with LDLR^{-/-} mice (Fig. 6A). Despite the lower plasma lipid levels, LDLR^{-/-}/VDR^{-/-} mice developed significantly larger lesions at the IA site compared to LDLR^{-/-} mice after 8-week dietary treatment (Fig. 6B and

6C). Within the AR site, the sinus lesions not associated with a coronary artery (NC) were also significantly larger in LDLR^{-/-}/VDR^{-/-} mice (Fig. 6D). At week 12, the lesion sizes were indistinguishable between LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice in the context of lower plasma cholesterol levels in LDLR^{-/-}/VDR^{-/-} mice (Fig. 6A). These observations indicate that VDR deletion accelerates atherogenesis in the early course, suggesting that VDR inhibits the initiation of atherosclerosis more than its progression. In view of the lower plasma cholesterol levels, known to be the major risk factor for atherosclerosis (56), however, the inhibitory role of VDR in atherogenesis might well be underestimated in the LDLR^{-/-}/VDR^{-/-} mice.

Consistent with the accelerated atherosclerotic phenotype, LDLR^{-/-}/VDR^{-/-} mice expressed significantly higher levels of adhesion molecules ICAM-1 and E-selectin in the aorta compared to LDLR^{-/-} mice after 4-week HFHC diet feeding. MCP-1, a chemokine that plays a key role in monocyte recruitment into the intima of vascular wall, was also markedly up-regulated in the aorta of LDLR^{-/-}/VDR^{-/-} mice. Peritoneal macrophages isolated from LDLR^{-/-}/VDR^{-/-} mice showed significantly higher ICAM-1 and P-selectin expression after being stimulated with acetylated LDL (acLDL). MCP-1, IL-1, INF and MMP-9 were also more elevated in LDLR^{-/-}/VDR^{-/-} peritoneal macrophages compared with LDLR^{-/-} counterparts. Increased adhesion molecules and pro-inflammatory factors in the aorta and macrophages are predicted to promote lesion initiation.

We also observed that, when stimulated with acLDL, cholesterol uptake by LDLR^{-/-}/VDR^{-/-} macrophages was more robust compared to LDLR^{-/-} macrophages. Consistent with this observation, SR-A1 and CD36, membrane proteins that mediate cholesterol influx, were markedly up-regulated in LDLR^{-/-}/VDR^{-/-} macrophages. SR-A1 elevation was also detected in the aorta isolated from LDLR^{-/-}/VDR^{-/-} mice after 4-week HFHC diet feeding. Cholesterol accumulation was also increased in VDR^{-/-} macrophages, suggesting that the VDR regulates macrophage cholesterol influx independent of the LDLR. In contrast, cholesterol efflux was not different between LDLR^{-/-}/VDR^{-/-} and LDLR^{-/-} macrophages. As macrophage conversion to foam cells is a critical step in atherogenesis, these observations suggest that the VDR signaling inhibits foam cell formation by suppressing cholesterol influx in macrophages. Taken together, we speculate that macrophage dysfunction plays a key role in promoting and accelerating atherosclerosis in LDLR^{-/-}/VDR^{-/-} mice.

T and B cell-Independent Anti-atherosclerotic Activity of VDR Signaling

As the difference in plasma lipid levels may potentially confound the animal data, we used bone marrow (BM) transplantation to explore the role of macrophage VDR signaling in the regulation of atherogenesis. We observed that LDLR^{-/-} recipient mice transplanted with LDLR^{-/-} BM or LDLR^{-/-}/VDR^{-/-} BM showed no significant difference in plasma lipids after 8-week HFHC dietary treatment (Fig. 7A), but the lesions were significantly larger at the AA site in LDLR^{-/-}/VDR^{-/-} BM transplanted (BMT) recipient mice compared to LDLR^{-/-} BMT recipient mice (Fig. 7B). The lesions at the other sites were not significantly different between these two BMT recipients. To eliminate the confounding effects of T and B lymphocytes on atherosclerosis, we performed another BM transplantation experiment in which LDLR^{-/-} recipients were transplanted with BM derived from Rag-1^{-/-} or Rag-1^{-/-}/VDR^{-/-} mice. Because Rag-1^{-/-} mice do not form mature T and B lymphocytes (79), the resulting Rag- BMT and Rag-1^{-/-}/VDR^{-/-} BMT mice are deficient in functional T and B cells. After 8-week HFHC diet feeding, no significant difference was detected in plasma lipid levels between Rag-1^{-/-} BM and Rag-1^{-/-}/VDR^{-/-} BMT mice (Fig. 7C); however, atherosclerotic lesions at the 1A and AA sites were significantly larger in Rag-1^{-/-}/VDR^{-/-} BMT mice (Fig. 7D). These data strongly suggest that leukocyte VDR signaling, likely macrophage VDR, plays a key role in the regulation of atherogenesis.

Regulation of Local Renin-Angiotensin System by Macrophage VDR Signaling

We have shown that renin and angiotensinogen (AGT) are directly regulated by $1,25(\text{OH})_2\text{D}_3$ (80–82). Given the promoting role of the RAS in atherosclerosis, we assessed the local RAS status in the aorta and macrophages. We observed that the expression of renin, AGT and AT1 receptor was markedly elevated in peritoneal macrophages isolated from VDR mice (Fig. 8A and 8B). Similar up-regulation of renin, AGT and AT1 receptor was also seen in peritoneal macrophages obtained from $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice at baseline or after being stimulated with acLDL (Fig. 8C and 8D). Renin receptor was also increased in stimulated $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ macrophages (Fig. 8D). In the aorta obtained from 4-week HFHC diet-fed $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice, renin was dramatically up-regulated compared to $\text{LDLR}^{-/-}$ controls (Fig. 8E and 8F). Similar renin up-regulation was also seen in the aorta from VDR^{-/-} mice. Importantly, immunostaining showed co-localization of marked renin elevation and macrophages within the atherosclerotic lesion in $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice (Fig. 8G). Together these data demonstrate that in the absence of VDR signaling, the local RAS is up-regulated in macrophages and aorta. Given the critical role of Ang II in atherosclerosis, this local RAS activation within the aorta likely promotes lesion progression.

To address whether renin up-regulation indeed contributes to the increased atherosclerosis seen in $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice, we treated these mice with aliskiren, a specific renin inhibitor previously shown to reduce atherosclerosis (71). Indeed, 8-week aliskiren treatment not only significantly reduced the size of atherosclerotic lesions and but also eliminated the difference between $\text{LDLR}^{-/-}$ and $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice under the atherogenic dietary condition. We also performed similar aliskiren treatment on $\text{LDLR}^{-/-}$ recipients transplanted with Rag-1^{-/-} BM or Rag-1^{-/-}/\text{VDR}^{-/-} BM on the HFHC diet. Again 8-week aliskiren administration completely eliminated the difference in atherosclerotic lesion size between Rag-1^{-/-} BMT and Rag-1^{-/-}/\text{VDR}^{-/-} BMT mice. These data provide strong evidence demonstrating that activation of the local RAS in leukocytes, most likely in macrophages, plays a key role in promoting atherosclerosis in the absence of vitamin D-VDR signaling.}}

Anti-Atherosclerotic Mechanism of Macrophage VDR Signaling

These studies demonstrate that the VDR signaling, most probably macrophage VDR signaling, inhibits atherogenesis. Although the relative lower lipid profiles caused by VDR deletion do not favor atherogenesis, several lines of evidence suggest that VDR deletion renders mice prone to atherosclerosis: The aorta from $\text{LDLR}^{-/-}/\text{VDR}^{-/-}$ mice exhibits up-regulated adhesion molecules and chemokines early in the course of atherosclerosis; Macrophages with VDR deletion are more inflammatory in terms of cytokine/chemokine production. Moreover, VDR^{-/-} macrophages show increased cholesterol uptake. Because cholesterol efflux is unaltered, increased influx will lead to intracellular cholesterol accumulation, suggesting that VDR signaling inhibits foam cell formation as reported previously in macrophages from type 2 diabetes patients (76).

We performed BM transplantation to eliminate the confounding effect resulting from the difference in plasma lipid levels. In this experiment, plasma lipid levels were comparable regardless of the status of VDR in BM-derived cells, but the absence of the VDR in these donor cells was still associated with an increment in atherosclerosis. In another BM transplantation experiment to eliminate the potential contribution of T and B cells in VDR-mediated inhibition of atherosclerosis, the increased atherosclerosis phenotype was reproduced. This points to leukocytes, likely macrophages, as the major and most probable site of action of vitamin D signaling in modulating atherogenesis. Consistent with an anti-atherosclerotic role of macrophage VDR, we found that oxidized LDL markedly suppressed $1,25(\text{OH})_2\text{D}_3$ -induced VDR expression in peritoneal macrophages. We speculate that high

levels of modified LDL are able to diminish the protective effect of VDR in macrophages, allowing atherogenesis to proceed.

Our data suggest that the local RAS in macrophages is a main target in vitamin D inhibition of atherosclerosis. All RAS components are found in the vascular wall (83–87) and in atherosclerotic lesions (88, 89). These RAS components co-localize with macrophages, particularly under hypercholesterolemic conditions (68, 90), suggesting that exposure to high levels of lipids activates the macrophage RAS leading to increased local Ang II generation in atherosclerotic lesions. Ang II promotes atherosclerosis at all stages of the atherosclerosis course independent of its effect on blood pressure (90, 91), including induction of endothelial dysfunction and vascular inflammation (92) and LDL oxidation (93), and promotion of monocyte recruitment (94–97), foam cell formation (98), vascular smooth muscle cell proliferation (99–101) and plaque instability (102, 103). We observed marked up-regulation of the RAS in peritoneal macrophages, the aorta and macrophages/foam cells within the atherosclerotic lesions in the absence of VDR. Importantly, we showed that blockade of renin by aliskiren ameliorated atherosclerosis in two animal models, suggesting that the local RAS in VDR-null leukocytes/macrophages is most likely responsible for the increase in atherosclerosis. These findings, together with the well established role of macrophage renin in atherosclerosis promotion (71), prompt us to infer that VDR signaling in leukocytes, likely macrophages, inhibits atherosclerosis at least in part by suppressing the local RAS in macrophages (Fig. 9).

Conclusion

The notion that vitamin D hormone as a negative endocrine regulator of the RAS has been well-established with support from epidemiological, physiological, biochemical and molecular data reported in recent years. This discovery reveals an important physiological function of the vitamin D endocrine system. Long-term vitamin D-deficiency, therefore, can lead to activation of the RAS, and vitamin D can target the RAS physiologically and pharmacologically. The broad functionalities of the RAS predict that vitamin D may regulate the RAS in renal and cardiovascular protection. Consistent with this view, our recent investigations demonstrate that podocyte VDR signaling protects against diabetic renal injury by suppressing the local RAS in podocytes and thus blocking podocyte apoptosis, and macrophage VDR signaling inhibits atherosclerosis by targeting the local RAS in macrophages, which plays key roles in promoting atherosclerotic lesion development. These findings have invaluable pathophysiological and therapeutic implications. They provide mechanistic insights into the everincreasing epidemiological and clinical evidence linking vitamin D deficiency to renal and cardiovascular problems, and also set molecular bases to explore therapeutic use of vitamin D and its analogs in the prevention and intervention of these diseases.

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Figure 1.

Podocyte VDR signaling ameliorates albuminuria and glomerular injury in diabetes. (A) Urinary albumin to creatinine ratio (ACR) over time. ND, non-diabetic; WT, wild-type; Tg, transgenic; V, vehicle; Dox, doxercalciferol. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. WT+V. (B) Glomerular VDR protein levels in WT and Tg mice in non-diabetic and diabetic states. (C) Immunostaining of kidney sections with anti-WT1 (red) or anti-synaptopodin (Green) antibodies. (D) Semi-quantitation of WT1-positive cells per glomerulus (Glom). (E) PAS staining of kidney sections showing accumulation of extracellular matrix in the glomeruli. (F) Glomerulosclerotic (GS) score based on PAS staining. * $P < 0.05$; *** $P < 0.001$ vs. WT+V or WT+Dox. (G) Fibronectin (FN) and nephrin levels in glomerular lysates. (H) qPCR

quantitation of slit diaphragm proteins podocin, CD2AP, nephrin and ZO-1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2.

Podocyte VDR signaling inhibits podocyte apoptosis. (A) TUNEL staining of kidney sections showing the glomerular TUNEL-positive podocytes. (B) Semi-quantitation of TUNEL positive cells per 100 glomeruli (Glom). ** $P < 0.01$; *** $P < 0.001$. (C) Hoechst 33342 staining of podocyte nuclei. Arrows indicate condensed nuclei. (D) Semi-quantitative data showing percentage of condensed nuclei under different cultural conditions.

*** $P < 0.001$ vs. the rest. (E) DNA fragmentation assays. (F) Western blots showing full-length and cleaved 17 kD caspase 3. (G) Semi-quantitation of condensed nuclei showing that p38 inhibition by SB203580 attenuates podocyte apoptosis induced by HG or Ang II. ** $P < 0.01$; *** $P < 0.001$. LG, low glucose; HG, high glucose; VD, 1,25-dihydroxyvitamin D; M, mannitol; SB, SB203580; Ang II, angiotensin II; Los, losartan.

Figure 3.

Podocyte VDR signaling inhibits the renin-angiotensin system (RAS). (A and B) Western blots (A) and quantitation (B) showing renin protein levels in glomerular lysates obtained from different mice. * $P < 0.05$ vs. WT, Tg and WT+Dox; ** $P < 0.01$ vs. the rest. (C) RT-PCR analysis of the RAS components in podocyte cultures; (D) Cellular lysate renin activity in podocytes cultured in different conditions; (E) Ang II levels in the media of podocyte cultures. ** $P < 0.01$; *** $P < 0.001$ vs. the rest.

Figure 4.

The hVDR transgene rescues VDR-null mice from developing severe renal injury. (A) Glomerular VDR protein levels in VDRKO and Tg-KO mice; (B) Urinary albumin to creatinine ratio (ACR) in different mice as indicated; * $P < 0.05$, *** $P < 0.001$ vs. VDRKO. (C) Synaptopodin immunostaining of kidney sections from different mice. (D) Semi-quantitation of WT1-positive cells per glomerulus; ** $P < 0.01$. (E) PAS staining of kidney sections. (F) Glomerulosclerotic (GS) score of kidney sections; * $P < 0.05$; (G) Electron microscopic examination of glomerular filtration barrier. *Arrows* indicate the width of the GBM; *Arrowheads* indicate effacement of foot processes. (H) GBM thickness. * $P < 0.05$ vs. the rest; (I) Glomerular levels of FN; (J) Glomerular levels of nephrin. (K) qPCR

quantitation of renin transcript in whole kidney or in glomeruli of different mice. ** $P < 0.01$, *** $P < 0.001$.

Figure 5.

Renoprotective mechanisms of podocyte VDR signaling. Hyperglycemia activates p38 MAPK- and ERK-mediated signaling pathways to induce podocyte apoptosis. 1,25-dihydroxyvitamin D (VD) inhibits the induction of angiotensinogen and renin by high glucose, leading to suppression of Ang II production. VD may also suppress p38 or ERK activation via an Ang II-independent mechanism. VD up-regulation of nephrin also contributes to the protection of podocytes.

Figure 6.

Anti-atherosclerotic activity of VDR signaling. (A) Plasma total cholesterol (*left panel*) and triglyceride (TG, *right panel*) levels in LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice fed the HFHC diet for 8 or 12 weeks (W); (B) Atherosclerotic lesion sizes at the four vascular sites (IA, AA, DA and AR) at week 8 of diet; (C) Lesion morphology at the IA site at week 8; (D) Lesion sizes in the three sinuses of the AR at 8 weeks. *P<0.05, ***P<0.001 vs. LDLR^{-/-}.

Figure 7.

Role of leukocyte/macrophage VDR signaling in atherosclerosis. (A and B) Effects of VDR-deleted bone marrow transplantation (BMT) on atherosclerosis. LDLR^{-/-} mice were transplanted with LDLR^{-/-} bone marrow (LDLR^{-/-} BMT) or with LDLR^{-/-}/VDR^{-/-} bone marrow (LDLR^{-/-}/VDR^{-/-} BMT). Atherosclerosis was induced by 8-week HFHC feeding. (A) Plasma total cholesterol (Chol) and triglyceride (TG) levels; (B) Atherosclerotic lesion sizes at the four vascular sites (IA, AA, DA and AR). (C and D) Effect of VDR-deleted, T and B cell-deficient bone marrow transplantation on plasma lipids (C) and atherosclerosis (D). LDLR mice transplanted with Rag-1 bone marrow (Rag-1 BMT) or with Rag-1^{-/-}/VDR^{-/-} bone marrow (Rag-1^{-/-}/VDR^{-/-} BMT) were fed the HFHC diet for 8 weeks.

Figure 8.

Effects of VDR deletion on the local renin-angiotensin system (RAS) in macrophages and aorta. (A and B) mRNA (A) and protein (B) levels of RAS components in peritoneal macrophages isolated from WT and VDR^{-/-} mice; (C and D) qPCR quantitation of RAS components in peritoneal macrophages isolated from LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice at baseline (C) or after acLDL stimulation (D); (E and F) RT-PCR amplification (E) and quantitation (F) of renin transcripts in the aorta from LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice. (G) Atherosclerotic lesions from LDLR^{-/-} and LDLR^{-/-}/VDR^{-/-} mice stained with anti-MOMA-2 (red) and anti-renin (green) antibodies. Note that renin is co-localized with

macrophages (yellow), and LDLR^{-/-}/VDR^{-/-} macrophages have higher renin levels compared with LDLR^{-/-} macrophages.

Figure 9.

Anti-atherosclerotic mechanisms of macrophage VDR signaling. Macrophage vitamin D-VDR signaling suppresses the local RAS leading to reduction in local Ang II production. Ang II promotes atherosclerosis by up-regulating adhesion molecule expression, stimulating LDL oxidation, macrophage lipid uptake and foam cell formation, and inducing smooth muscle cell (SMC) migration and proliferation.