Vitamin D Attenuates Kidney Fibrosis via Reducing Fibroblast Expansion, Inflammation, and Epithelial Cell Apoptosis

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Kidney fibrosis is the common final pathway of chronic kidney diseases (CKD). It is characterized by myofibroblast formation, inflammation, and epithelial architecture damage. Vitamin D is known as a renoprotective agent, although the precise mechanism is not well understood. This study aimed to elucidate the effect of vitamin D in fibroblast expansion, inflammation, and apoptosis in kidney fibrosis.

We performed unilateral ureteral obstruction (UUO) in male Swiss-Webster background mice (3 months, 30–40 grams) to induce kidney fibrosis. The mice (n=25) were divided into five groups: UUO, 3 groups treated with different oral vitamin D doses (0.125 µg/kg (UUO+VD1), 0.25 µg/kg (UUO+VD2), and 0.5 µg/kg (UUO+VD3)), and a Sham operation (SO) group with ethanol 0.2% supplementation. We sacrificed the mice on day14 after the operation and harvested the kidney. We made paraffin sections for histological analysis. Tubular injury and fibrosis were quantified based on periodic acid-Schiff (PAS) and Sirius Red (SR) staining. Immunostaining was done for examination of myofibroblasts (αSMA), fibroblasts (PDGFRβ), TLR4, and apoptosis (TUNEL). We did RNA extraction and cDNA for Reverse transcriptase PCR (RT-PCR) experiment for measuring MCP-1, ICAM-1, TLR4, and collagen 1 expression. TGFβ1 level was quantified using ELISA.

We observed a significantly lower levels of fibrosis (p<0.001), tubular injury scores (p<0.001), and myofibroblast areas (p<0.001) in the groups treated with vitamin D compared with the UUO group. The TGFβ1 levels and the fibroblast quantifications were also significantly lower in the former group. However, we did not find any significant difference among the various vitamin D-treated groups. Concerning the dose-independent effect, we only compared the UUO+VD-1 group with SO group and found by TUNEL assay that UUO+VD-1 had a significantly lower epithelial cell apoptosis. RT-PCR analysis showed lower expression of collagen1, as well as inflammation-mediation expression (MCP-1, ICAM-1, TLR4) in the UUO+VD-1 group compared with the SO group.

Vitamin D reduces kidney fibrosis through inhibition of fibroblast activation, and ameliorates epithelial cell architecture.

INTRODUCTION

Kidney fibrosis is a final common pathway of chronic kidney diseases (CKD), which is characterized by myofibroblast formation and activation. These cells serve not only as matrix-producing cells but also as a key modulators of disease progression (1). Kidney fibrosis is marked by the improvement of fibrogenesis markers, especially transforming growth factors-β1 (TGF-β1), which plays a role in the underlying biomolecular pathomechanism of progressive kidney diseases (2). There are three local events needed to generate α-SMA-positive myofibroblasts: TGF-β1; the presence of ECM protein such as EDA, a splice variant of fibronectin; and high extracellular stress and cell remodeling activity (3).

Unilateral ureteral obstruction (UUO) is widely used renal tubulointerstitial fibrosis model (4, 5). UUO is a good model of renal tubulointerstitial fibrosis because of the inexistence of exogenous toxins, the minimization of a “uremic” environment, and the ability to use the contralateral kidney as control (6). Myofibroblast formation is the hallmark of UUO and kidney fibrosis. Many studies have revealed that numerous cells may

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contribute as sources of myofibroblasts in UO, such as epithelial cells through EMT (7), bone marrow-derived cells, endothelial cells, pericytes, or perivascular fibroblasts (8), and extrarenal-origin fibroblasts (9).

Vitamin D is known to have not only a role in bone mineral metabolism but also a renoprotective effect (10). Some mechanisms of vitamin D action targeting fibrosis prevention have been investigated (11, 12), but further research is necessary to clarify the precise action mechanism. Vitamin D has been known to induce apoptosis in some cancers (13, 14). Nevertheless, its antiapoptotic effect in renal epithelial cells would be interesting to elucidate. Therefore, in this study, we focused on elucidating the effect of vitamin D on renal interstitial cells, especially fibroblast activation, inflammation, and renal epithelial cells apoptosis, as an underlying mechanism of renal fibrosis.

MATERIALS AND METHODS

Animal Subjects

Experiments were done after approval by the Ethical Committee of the Faculty of Medicine, GadjahMada University. Male Swiss-Webster mice (n = 25) age 8 weeks old, with 30–40 gr body weights were obtained from the Experimental Animal Care Unit (UPHP) LPPT of Gadjah Mada University. Mice were housed in a cage owned by the Department of Anatomy, Faculty of Medicine, Gadjah Mada University, with a light-dark cycle of 12:12 hours. We performed UUO/ureter ligation to induce kidney fibrosis. Briefly, mice were anesthetized with sodium pentobarbital (0.1 mg/kg body weight). The abdomen was opened in the right-flank region. The right ureter was visualized and then double ligated in the pelvico-ureter junction and then cut between the ligation sides. A sham operation (SO) procedure was used for the control group with the same procedure except for ligating and cutting the ureter. Subjects were divided into five groups, that is, SO (sham-UUO + ethanol 0.2%), UUO (UUO + ethanol 0.2%), UUO+VD1 (UUO + vitamin D 0.125 µg/kg), UUO+VD2 (UUO + vitamin D 0.25 µg/kg), and UUO+VD3 (UUO + vitamin D 0.5 µg/kg). Vitamin D treatment was done with intraperitoneal injection of Cholecalciferol that was diluted with ethanol 0.2%. Mice got standard chow and free access to water ad libitum. Mice were terminated at day 14 after the operation.

Kidney harvesting

For sacrifice, the mice were anesthetized with pentobarbital (60 mg/kg ip); then the abdomen and thorax were opened. Organs were perfused with 0.9% NaCl from the left ventricle. Right kidney tissues were harvested, and half was kept in RNA later® for ELISA quantification and RNA extraction. The other half was fixed in 4% PFA in PBS for 24 hour, and paraffin was used in the embedded tissue process.

Histological analysis, immunostaining, and TUNEL

Paraffin sections with 4-mm thickness were analyzed. Paraffin sections were deparaffinized, and stained with periodic acid-Schiff (PAS) to evaluate tubular injury, and Sirius Red (SR) to quantify the tubular injury score and fibrosis interstitial fraction area. Immunohistochemical (IHC) staining was done for the following antibodies: PDGFβ for fibroblasts (Abcam, 1:200; ab32570), α-SMA (1:500; Sigma, A2547), and Toll-Like Receptor 4 (TLR4, 1:200; Bioss, bs1021R). Briefly, after deparaffinizing, paraffin sections were heated in citrate buffer (10 mM Sodium Citrate, 0.05% Tween20 pH 6) and then incubated in 3% H2O2 in PBS. Antibodies were incubated overnight after blocking with 3% bovine serum albumin in PBS. The following secondary antibodies were used and incubated for 1 hour: Anti-Rabbit Dako Envision labeled Polymer-HRP (DAKO, K4000) for PDGFβ and TLR4, Histofine anti-mouse (Nichirei, 414171F) for α-SMA staining. TUNEL staining was done using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Millipore, S700), based on the instructions in the kit. Quantification of fibrosis and myofibroblast fraction area was done using ImageJ software, with 15 fields in each sample and with 400x magnification. Meanwhile, fibroblast and apoptosis positive epithelial cells number were counted with 12–15 fields in each sample, with 400x magnification.

Tubular Injury Score and interstitial fibrosis fraction area quantification

Tubular injury was assessed by PAS staining, based on the previously described method (3). Scoring was done by grading tubular dilatation, epithelial simplification, and brush border loss in 15 randomly chosen, nonoverlapping fields (200 magnification). The lesions were graded on a scale from 0 to 4: 0, normal; 1, mild, involving less than 25% of the cortex; 2, moderate, involving 25% to 50% of the cortex; 3, severe, involving 50% to 75% of the cortex; and 4, extensive damage, involving more than 75% of the cortex. Area fraction of collagen was analyzed using Image J software on 15 nonoverlapping files. The measurement result was expressed as a percentage (%).

TGF-β1 Analysis by ELISA

ELISA was used for quantification of the TGF-β1 levels in kidney tissue. Protein extraction and quantification was done based on the kit’s instruction (Elabscience, E-EL-M0051). The optical density value was read from a microplate reader at 450 nm wavelength. Afterward, a standard curve of a linear equation was made so that the level of TGF-β1 was set on pg/mL.
Reverse Transcriptase PCR (RT-PCR)

RNA was extracted from kidney tissue using Trizol (Invitrogen, 1559-018, Paisley, UK). cDNA was synthesized using ReverTra-Ace (TOYOBO Co., Ltd, TRT-101x10). Reverse transcriptase PCR (RT-PCR) was done for examining the expression of following genes: MCP-1 (forward, 5'-GGCATCACAGTCGAGTCACA-3'; reverse, 3'-CTACAGACAACCCACCTCAAGCCTTC-5'), TLR4 (forward, 5'-GGGCCTAAACCCAGTCTGTTTG-3'; reverse, 3'-GCCCGGTAAAGTCATGCTA-5'), ICAM-1 (forward, 5'-CAATTCAACTGAATGCCTCAAGCCTC-3'; reverse, 3'-CAAGCAGTCCGTCTGGTCA-5'), collagen 1 (forward, 5'-ATGCCGACCTCAAGATG-3'; reverse, 5'-TGAGGCAGACAGCCTGAGTA-3'), and GAPDH (forward, 5'-TTGCTGGTGAAGTCGCAGGAG-3'; reverse, 5'-TTGTGTCCGTGGATGCTGA-3') was used as reference.

Statistical Analysis

Data were presented as mean±SEM for the area fraction of collagen, and as median (min–max) for TGF-β1 levels and the area fraction of myofibroblasts. The level of TGF-β1 and the area fraction of myofibroblasts were analyzed by using Kruskal–Wallis and Mann–Whitney tests. The average of area fraction of collagen, which has normal data distribution, was analyzed by one-way ANOVA test and continued with Post Hoc test.

RESULTS

Vitamin D supplementation attenuated tubular injury, interstitial fibrosis, and myofibroblast fraction area

UOO was found to induce interstitial fibrosis and tubular injury dramatically, based on SR and PAS staining quantification. These findings were followed by an increasing of serum creatinine level. The findings demonstrated rapid disruption of kidney architecture caused by fibrosis. Our quantification showed significantly lower fibrosis fraction areas, tubular injury scores, and serum creatinine levels in groups with vitamin D supplementation (UOO+VD1, UOO+VD2, and UOO+VD3). However, vitamin D groups still had significantly higher incidence of fibrosis and tubular injury, as well as higher creatinine levels, compared with the levels in the SO group (Figure 1). We also did not find any difference in fibrosis, tubular injury scores, and serum creatinine levels among the three different vitamin D supplementation groups.

![Figure 1](image-url)

Figure 1. A. Representative picture of Sirius Red and PAS staining to show fibrosis and tubular injury. B-D. Quantitative analysis of creatinine, interstitial fibrosis area fraction, and tubular injury score.
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**Vitamin D reduced myofibroblasts through reducing TGF-β1 level and fibroblast expansion**

Myofibroblast fraction-area quantification revealed reduction of the fraction in vitamin D groups, whereas there were no significant differences among the vitamin D groups. Further, TGF-β1 level was also lower in groups with vitamin D supplementation (p<0.001 vs UUO group). These finding were associated with lower fibroblast cell number in the UUO+VD1, UUO+VD2, and UUO+VD3 groups compared with the UUO group (p<0.001, Figure 2). Statistical analysis disclosed no differences of TGF-β1 level, myofibroblast fraction area, and fibroblast cell number among vitamin D groups. However, statistical analysis showed significant difference between the SO and vitamin D groups tests (Figure 2).

![Graph showing TGF-β1 levels and myofibroblast fraction area](image)

**Lower epithelial cell apoptosis and inflammation in vitamin D groups**

Observing that there was no dose dependence in the effect of vitamin D (Figure 1&2), we focused on the UUO+VD1 group to elucidate the effect of vitamin D supplementation in epithelial cell apoptosis and inflammation. Assessment of apoptosis staining using TUNEL revealed an increasing number of apoptotic epithelial cells because of UUO. This was associated with upregulation of inflammatory mediator expressions such as ICAM-1, MCP-1, and TLR-4. Vitamin D induction significantly reduced the number of apoptotic epithelial cells and the inflammatory mediator (Figure 3). Immunostaining of TLR-4 showed positivity in the epithelial and interstitial area. Those positive areas were reduced in the UUO+VD1 group.

**DISCUSSION**

UUO is considered a relevant model for kidney fibrosis. Kidney fibrosis is characterized by interstitial fibrosis with myofibroblast formation and renal architecture damage (8). UUO induces profibrotic growth factors such as transforming growth factor -β1, and this profibrotic signaling inhibition attenuates kidney fibrosis in the UUO model (15). In this study, vitamin D reduced not only interstitial fibrosis but also tubular injury, whereas word vitamin D ameliorated renal architecture (Figure 1). The myofibroblast is a key cellular mediator of...
fibrosis, and it serves as the primary collagen producing cell (1). Understanding the origin of this cell is believed to be one of the best approaches for fibrosis therapy. There are various cells that have been proposed as sources of myofibroblasts in kidney fibrosis, including epithelial cells through the epithelial to mesenchymal transition (7), fibrocytes (16), endothelial cells through the endothelial to mesenchymal transition (17), renal interstitial cells such as pericytes or perivascular fibroblasts (18), and fibroblasts (9).

To elucidate a novel role of vitamin D in renal interstitial cell activation as a myofibroblast source, we used immunostaining of platelet-derived growth factor receptor β (PDGFRβ) to mark renal interstitial cells. Other studies also used this marker to observe two renal interstitial cells, pericytes and fibroblasts (9, 18). There is still a limitation in the study because there is no specific marker for both of the cells (19). The difference between the cells is based on the anatomical location of the cells in the vessel. Pericytes are in close contact with endothelial cells (EC) in microvessels, whereas fibroblasts are not in close contact with EC (8). This topic, however, is beyond the scope of this study. We used PDGFRβ positive cells to refer to renal interstitial cells, although we could not differentiate whether those cells were fibroblasts or pericytes. Vitamin D supplementation could reduce the number of PDGFRβ positive cells number and reduce the myofibroblast fraction area; this is assuming that the reduction of myofibroblasts was through attenuating fibroblast activation (Figure 2). We did not find any difference among the three different vitamin D supplement dose groups. These findings might be associated with the significantly lower levels of TGF-β1 protein in three groups of the vitamin D (Figure 2). The effect of vitamin D on TGF-β1 has been observed in other studies, although some studies used the other form of vitamin D. Paracalcitriol inhibits the formation of TGF-β1 and reduces its receptor expression (20). Vitamin D also inhibits the signal transduction of TGF-β-Smad by inhibiting pSmad3 recruitment to the promoter area at the TGF-β genes target, that is, serpine1 (code of plasminogen activator inhibitor-1), acta2 (code of α-SMA), and Col1A1 (code of type-1 collagen) (11). Vitamin D also induces hepatocyte growth factors (HGF) through interaction between the vitamin D receptor and VDRE with the HGF promoter region. HGF inhibits myofibroblast activation and epithelial to mesenchymal transition (EMT) (12). Through this study, we proposed

Figure 3. A. Quantification of apoptotic epithelial cell number in each group showed a reduction in the UUO+VD group (0.125µg/kg body weight dose). B. Representative picture of TUNEL and TLR4 immunostaining. C-D. Reverse transcriptase PCR (RT-PCR) measurement of collagen 1, ICAM-1, MCP-1 and TLR4, and densitometry analyzes using Image J software. ***p<0.001 vs SO, # p<0.05 vs UUO group, **p<0.01 vs SO, ##p<0.01 vs UUO.
the influence of vitamin D in renal interstitial cell activation. In vitro study had shown the contribution of vitamin D in the inhibition of collagen 1 and fibronectin synthesis in TGF-β-treated lung fibroblasts (21). Further study is needed to examine the effect of vitamin D in renal fibroblasts to confirm our findings.

Regarding the independent dose of vitamin D effect in this study, we focused on observing inflammation and epithelial cell apoptosis in the UUO+VD1 group. Those independent effects might be due to similar vitamin D levels in serum between the three vitamin D groups, although we did not measure the vitamin D serum concentration. TUNEL assay revealed that UOO induced an increase of apoptosis in epithelial cells. Vitamin D-treated groups had significantly lower numbers of apoptotic epithelial cells (Figure 3). Apoptosis is one of the mechanism of renal tubular damage in kidney fibrosis (22). The effect of vitamin D in the reduction of epithelial cell apoptosis might be modulated by TGFβ1-Smad3 signaling, using a non-classical pathway. It has been proven that vitamin D suppresses renal fibrosis without activating vitamin D receptor (VDR)-mediated transcription (11). TGFβ1-Smad3 signaling deficiency could prevent renal fibrosis through apoptosis and inflammation inhibition (23). Other studies might have reached opposite findings because of the proapoptotic effect of vitamin D in the breast cancer cell line (14, 13). Other mechanisms might contribute to the antiapoptotic effect, such as FGF-23-Klotho (24) signaling in this antiapoptotic effect of vitamin D.

Further, we also revealed the anti-inflammatory and anti-fibrosis effects of vitamin D through downregulation of proinflammatory mediators, such as Toll-Like Receptor 4 (TLR4), Inter Cellular Adhesion Molecule 1 (ICAM1), and Monocyte Chemoattractant Protein 1 (MCP1), in the UUO+VD1 group and compared them with those of the UUO group. During ischemia, TLRs signaling can be activated by endogenous ligands and trigger inflammatory response (25). Damaged tissues during UUO belong to endogenous ligands, which then activate TLRs (26). TLRs are expressed by the cells of the immune system, such as macrophages, DCs, neutrophils, B cells, and NK cells. TLRs may also be expressed in tissue by cell types, including kidney tubular epithelial cells and mesangial cells, in response to injury (27). Similar to those results, our immunostaining of TLR4 revealed damaged epithelial cells and inflammatory cells in interstitial space as the sources of TLR4 expression after UUO (Figure 3). Amelioration of epithelial cells and tubules architecture in vitamin D-treated groups might have reduced the damaged tissue/endogenous ligand for TLR4. This effect also leads to reduction of proinflammatory mediators such as MCP-1 and ICAM-1, and collagen 1 expression. We did not find a difference in the expression of TLR4 and collagen I between the SO and UUO+VitD group. Meanwhile, we found significant difference in PDGFR β positive cell number and interstitial fibrosis fraction area between UUO+VitD and SO group. The quantification of mRNA expression in this study used reverse transcriptase PCR (RT-PCR) rather than quantitative-RT-PCR (qRT-PCR), which might have affected the sensitivity of the quantification, which might be one of the limitations of the study. Analyses using qRT-PCR would be better and are suggested to increase the sensitivity in future tests.

Taken together, this study showed the beneficial effect of vitamin D supplementation in kidney fibrosis or end-stage renal disease (ESRD) conditions through reduction of fibroblast activation, inflammation, and epithelial cell apoptosis. Following study about adding vitamin D supplementary therapy to ESRD ambulatory therapy will be needed to adjust those effects in clinical use. We also need further study to understand the precise mechanism of vitamin D in both the classical and non-classical pathways in fibroblasts and other potential cells. In this study, we did not reveal the calcium level in serum. Calcium might play a role in the pathogenesis of renal injury: in the authors’ opinion, it is important to quantify the serum calcium level in the study of vitamin D and renal injury.

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