Reduction of Slit Diaphragm-associated Molecules by Sirolimus: Is it Enough to Induce Proteinuria?

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ABSTRACT

Sirolimus (SRL), a mammalian target of rapamycin inhibitor, is widely used in transplantation, but the mechanisms whereby it induces adverse effects, such as proteinuria and edema, remain unclear. To determine whether isolated SRL induces proteinuria or not, the authors intraperitoneally injected C57BL/6 mice with different doses of SRL (0 mg/[kg·d], 3 mg/[kg·d], 10 mg/[kg·d], or 30 mg/[kg·d]) for 24 days. Urinary albumin excretion was then quantified using a double-sandwich enzyme-linked immunosorbent assay, and serum creatinine levels were measured using a single dry-film chemistry auto-analyzer. The mRNA expression levels of various genes were also measured by polymerase chain reaction. Urinary albumin was not detected in the SRL-treated mice, but serum creatinine levels were found to increase dose-dependently and were significantly higher in the animals treated with 30 mg/kg of SRL than in untreated controls. Glomerular mRNA expression profiling showed down-regulations of podocyte-related genes (Wilms tumor 1, synaptopodin, nephrin, CD2-associated protein, and podocin) and of transforming growth factor-beta (a marker of fibrosis) in sirolimus-treated mice. In addition, expressions of the anti-apoptotic genes Bcl-2 and Bcl-xL were also down-regulated. Furthermore, the protein levels of these genes in mice kidney were also decreased by sirolimus. Although sirolimus treatment reduced the expressions of slit diaphragm–associated molecules and increased serum creatinine levels, it failed to induce proteinuria. Our findings indicate that proteinuria is not induced by isolated SRL treatment. Further studies are required to identify conditions in which sirolimus induces proteinuria.

PROTEINURIA, the most common clinical manifestation of glomerular diseases, is invariably associated with podocyte foot-process effacement and flattening. Podocytes are glomerular cells of the kidney and the integrities of their actin cytoskeletons to prevent the development of proteinuria [1], which occurs when damage to podocytes results in simplification of their foot processes, and subsequent podocyte loss due to detachment, necrosis, and apoptosis [2]. When this loss reaches a critical level, renal disease progresses with commensurate increases in serum creatinine level and loss of functional nephrons, which overloads remaining kidney cells, causing metabolic and mechanical stress and finally cell death [3].

High sirolimus (SRL) levels may induce focal segmental glomerulosclerosis de novo by reducing the expression of vascular endothelial growth factor [4]. SRL, a mammalian target of rapamycin inhibitor (mTORi), is known to induce immune tolerance in transplantation, and has been used

Supported by a faculty research grant (#6-2012-0021) at Yonsei University College of Medicine. Beom Seok Kim and Jae Geun Lee contributed equally to this work. Y. Cho is a research associate supported by Yonsei University Health System IACF (#2012-31-0585). Current address of Seung Hwang Song is Department of Surgery, College of Medicine, Ewha Womans University, Seoul, Republic of Korea.

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0041-1345/17
http://dx.doi.org/10.1016/j.transproceed.2017.03.021

clinically to prevent calcineurin inhibitor–induced nephrotoxicity and post-transplantation cancer development [5]. However, several adverse effects, including proteinuria, limit its routine application, and the mechanism by which SRL induces proteinuria remains unclear [6,7]. Because proteinuria is a major predictive factor of graft loss, this adverse event has attracted research attention [8]. However, little is known about the molecular mechanisms leading to proteinuria in renal transplantation patients receiving SRL. Therefore, to determine whether isolated SRL treatment induces proteinuria, we treated C57BL/6 mice with various doses of SRL.

MATERIALS AND METHODS

Mice

Intact male C57BL/6 mice (age, 8 weeks) were administered daily intraperitoneal injections of 0 mg/kg, 3 mg/kg, 10 mg/kg, or 30 mg/kg of SRL for 24 days. Animals were provided free access to tap water and a standard laboratory diet during the 8-week treatment period. Albuminuria was determined after housing mice in metabolic cages for 24 hours after 2 hours' to 3 hours' habituation. All animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine.

Glomerulus Isolation

At the end of the SRL treatment, kidneys were removed, decapsulated, and placed in ice-cold Hanks' balanced salt solution (HBSS 5.33 mmol/L potassium chloride, 0.44 mmol/L potassium phosphate monobasic, 138 mmol/L sodium chloride, 4 mmol/L sodium bicarbonate, 0.3 mmol/L sodium phosphate dibasic, and 5.6 mmol/L glucose). Cortices were carefully dissected and cut into small slices, and using a spatula, slices were passed through 250-μm, 150-μm, 125-μm, and 75-μm wire sieves to separate glomeruli. Glomeruli retained by the 75-μm sieve were washed using cold HBSS into 50-mL tubes; glomerular suspensions were then centrifuged and the pellets so obtained were re-suspended in HBSS. Sample purities were confirmed by microscopy to be ~80% on average with little variation between samples.

Urinary Albumin Measurement

Urinary albumin excretions were assessed by enzyme-linked immunosorbent assay using a mouse albumin enzyme-linked immunosorbent assay kit (AssayPro, St. Charles, Missouri, United States) according to the manufacturer's instructions.

Serum Creatinine Measurement

The blood was allowed to clot for 30 minutes and serum was separated by centrifugation. All samples were free of hemolysis and lipemia. Serum creatinine levels were measured using an enzymatic method and a dry chemistry analyzer (Fuji DRY-CHEM4000, Tokyo, Japan).

Total RNA Isolation and Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, United States) and reverse-transcribed using oligo-dT using M-MLV Reverse Transcriptase (Invitrogen). Synthesized cDNA samples were analyzed by polymerase chain reaction (PCR) using cDNA-specific primers for each gene: Wilms tumor-1 (WT-1): forward, 5′-AAAGTTTGCGCGCTCAGACGAA-3′, reverse, 5′-TGCAGTCAATCAGGTGTGCTGT-3′; synaptopodin: forward, 5′-TTCTGGCGCGCAACATCATCAA-3′, reverse, 5′-AGAAAGCAGCTCCACGGAAGTT-3′; nephrin: forward, 5′-TATGCTTGGCCATTGGAGGCAA-3′, reverse, 5′-TGCTGACGCAGGTCAAGTTCAA-3′; podocin: forward, 5′-AGTTCCTGGTGCAAACCACCAT-3′, reverse, 5′-ACTTTGGCCTGTCTTTGTGCCT; Bax: forward, 5′-GCTGATGGCAACTTCAACTG-3′, reverse, 5′-ATCAGCTCGGGCACTTTAG-3′; Bcl-xL: forward, 5′- CTGGTTGAGCCCATCTCTATT-3′, reverse, 5′-CTGACTCCAGCTGTATCCTTTC-3′; Bcl-2: forward, 5′-GAGCAGGTGCCTACAAGAAA, reverse, 5′-CTTTGTCCTCTGACTGGGTATG-3′; and GAPDH: forward, 5′-TCAACAGCAACTCCCACTCTTCCA-3′, reverse, ACCCTGTTGCTGTAGCCGTATTCA. PCR was performed using Bioneer Accupower PCR PreMix (Bioneer, Daejeon, Korea), and GAPDH was used as an internal control.

Western Blot Analysis

Protein extracts were prepared from kidney tissues using radioimmunoprecipitation assay (RIPA) buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, and 0.1% sodium dodecyl sulfate. Proteins were boiled for 5 minutes, separated by 10% to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted onto polyvinylidene difluoride membranes. Detection was performed using the following primary antibodies: Bcl-2 (1:1000), WT-1 (1:1000), podocin (1:1000), podoplanin (1:1000), CD2AP (Cell Signaling Technology, Beverly, Massachusetts, United States) and β-actin (1:10,000) (Sigma, St Louis, Missouri, United States); and an Immobilon Western Chemiluminescent HRP substrate kit (Millipore Corporation, Billerica, Massachusetts, United States).

Statistical Analysis

Results are presented as means ± SEMs. One-way analysis of variance with Tukey's post hoc test were used to determine the significances of differences between means. $P < .05$ was considered statistically significant, and the analysis was conducted using GraphPad Prism software (GraphPad Software, Inc., La Jolla, California, United States).

RESULTS

Changes in Transforming Growth Factor Beta 1, Slit-diaphragm–associated, and Apoptosis-related Gene Expressions in Mice Glomeruli After SRL Treatment

The glomerular mRNA expression of transforming growth factor beta (TGF-β, a marker of fibrosis) were lower in the intraperitoneally injected mice with 3 mg/kg, 10 mg/kg, or 30 mg/kg SRL (Fig 1A), and mRNA expression levels of slit-diaphragm (SD)–associated molecules, WT-1, synaptopodin, nephrin, CD2-associated protein (CD2AP), and podocin were decreased by SRL treatment (Fig 1B). Furthermore, the expressions of the anti-apoptotic genes Bcl2 and Bcl-xL, but not Bax, were reduced by SRL (Fig 1C).

Expression Levels of SD-associated Molecules After SRL Treatment in C57B/L6 Mice

After daily intraperitoneal injections of 3 mg/kg of SRL for 24 days, protein levels of WT-1, podocin, podoplanin, and