Blockade of endothelial-mesenchymal-transition by a Smad3 inhibitor delays the early development of streptozotocin-induced diabetic nephropathy

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**Objects:** A multicenter, controlled trial has revealed that early blockade of the renin-angiotensin system in patients with type 1 diabetes and normoalbuminuria did not retard the progression of nephropathy, suggesting that other mechanism(s) are involved in the pathogenesis of early diabetic nephropathy (DN). We have previously demonstrated that endothelial-mesenchymal-transition (EndoMT) contributes to the early development of renal interstitial fibrosis in streptozotocin (STZ)-induced diabetic mice independently of microalbuminuria. In the present study we hypothesized that blocking EndoMT reduces the early development of DN.

**Research Design and Methods:** EndoMT was induced in a mouse pancreatic microvascular endothelial cell line (MMEC) in the presence of advanced glycation end-products (AGEs) and in an endothelial lineage-traceable mouse line Tie2-Cre;Loxp-EGFP by administration of AGEs, with non-glycated mouse albumin serving as a control. Phosphorylated Smad3 was detected by immunoprecipitation/western blotting and confocal microscopy. Blocking studies using RAGE siRNA and a specific inhibitor of Smad3 (SIS3) were performed in MMECs and in STZ-induced diabetic nephropathy in Tie2-Cre;Loxp-EGFP mice.

**Results:** Confocal microscopy and real-time PCR demonstrated that AGEs induced EndoMT in MMECs and in Tie2-Cre;Loxp-EGFP mice. Immunoprecipitation/western blotting showed that Smad3 was activated by AGEs but was inhibited by SIS3 in MMECs and in STZ-induced DN. Confocal microscopy and real-time PCR further demonstrated that SIS3 abrogated EndoMT, reduced renal fibrosis and retarded progression of nephropathy.

**Conclusions:** EndoMT is a novel pathway leading to early development of DN. Blockade of EndoMT by SIS3 may provide a new strategy to retard the progression of DN and other diabetic complications.

**Diabetic Nephropathy (DN) is a major microvascular complication of both type I and type II diabetes. Increased glomerular basement membrane thickness, mesangial expansion, glomerular sclerosis and tubulointerstitial fibrosis are major features of DN. The severity of glomerulosclerosis and tubulointerstitial fibrosis are strong predictors of the progression to ESRD, making this an important therapeutic target.**

Current clinical treatment guidelines for DN include the control of hyperfiltration, microalbuminuria, systemic blood pressure and blood glucose (1). Multiple clinical trials have shown that blockade of the renin-angiotensin system (RAS) can improve renal function in late DN in patients with proteinuria, diabetes and reduced glomerular filtration rate (2-4). Recently however, a large scale, multicenter, controlled trial revealed that inhibition of the RAS in normotensive patients with type 1 diabetes and normoalbuminuria did not reduce the incidence of microalbuminuria or slow the decline of renal function, suggesting that the pathogenesis of early DN may differ from that of late diabetic renal disease (5).

Myofibroblasts are major contributors to extracellular matrix (ECM) accumulation in fibrotic disease, and their numbers inversely correlate with renal function in DN (6, 7). It is generally believed that myofibroblasts can be derived from renal fibroblasts, tubular epithelial cells, mesangial cells and bone marrow-derived cells. Recently, Zeisberg et al (8) showed that EndoMT contributed to cardiac fibrosis. They (9) further
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We recently demonstrated that 30% to 50% of fibroblasts in three different mouse models of renal disease (unilateral ureteral obstructive (UUO) nephropathy, streptozotocin (STZ)-induced diabetic nephropathy, and a mouse model of Alport syndrome) co-expressed the endothelial marker CD31 and the fibroblast/myofibroblast markers fibroblast specific protein-1 and/or α-smooth muscle actin (α-SMA). We also recently demonstrated that 10% to 24% of renal interstitial myofibroblasts in 1 and 6-month STZ-induced diabetic kidneys were of endothelial origin, revealing the existence of endothelial-mesenchymal-transition (EndoMT) in the development and progression of DN (10). However, it is unclear whether blockade of EndoMT can reduce renal fibrosis and retard the early development of DN.

There is increasing evidence of a causal role for advanced glycation end-products (AGEs) in the development of diabetic complications, including nephropathy and vasculopathy (11, 12). AGEs exert their effects through the formation of protein cross-links that alter the structure and function of ECM and by interacting with specific cell surface receptors (11). The best characterized AGE receptor is RAGE, although other AGE binding sites have been identified (12). Disruption of the RAGE gene ameliorates development and progression of DN (13). AGEs have also been shown to cause epithelial-mesenchymal transdifferentiation via RAGE in DN (14). It is unknown whether AGEs can induce EndoMT, and if they can, whether blockade of AGE-induced EndoMT can ameliorate the development and progression of diabetic renal fibrosis. The interaction of AGEs and RAGE on endothelial cells induces cellular oxidant stress and initializes serial signalling pathway activation, including the nuclear transcription factor-κB (NF-κB), extracellular signal regulated kinase 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), stress-activated protein kinase /c-Jun-N-terminal kinase (SAPK/JNK) and the small GTPase Ras, Rho-family small GTPase Cdc42 and Rac1 pathways (15-23). AGEs also induce rapid and transient activation of Smad2 and Smad3 in tubular epithelial cells, mesangial cells and vascular smooth muscle cells through RAGE-Smad2/3 crosstalk (24). Whether AGEs can induce Smad3 activation in renal endothelial cells and whether blockade of RAGE-Smad3 crosstalk abrogates AGE-induced EndoMT requires further investigation.

Smad3 plays an essential role in renal fibrosis. Smad3 conditional knockout mice have been shown to be resistant to STZ-induced renal fibrosis and tubulointerstitial fibrosis in UUO models (25-27). Recently, Jinnin et al. (28) showed that a specific inhibitor of Smad3 (SIS3) inhibited Smad3 phosphorylation and reduced a TGF-β1-induced fibrotic response in fibroblasts (10). We also demonstrated that SIS3 can abolish TGF-β1-induced EndoMT in mouse pancreatic microvascular endothelial cells (MMECs) (10). Whether SIS3 can inhibit AGE-induced EndoMT in vitro and in vivo remains to be explored.

In the present study we hypothesize that AGEs can induce EndoMT and that blockade of RAGE-Smad3 crosstalk not only abrogates AGE-induced EndoMT but also retards the early development of renal fibrosis in STZ-induced diabetic mice. AGE-induced EndoMT was examined in MMECs and in an endothelial lineage-traceable mouse line (Tie2-Cre;Loxp-EGFP) while the efficacy of SIS3 was tested in AGE-induced EndoMT in MMECs and in STZ-induced diabetic mice.

RESEARCH DESIGN AND METHODS

Animals. B6.Cg-Tg(Tek-cre)12F1v/J mice (Stock Number: 004128, 19) and B6.Cg-Tg(ActB-Bgeo/GFP)21Lbe/J mice (Stock Number 004178) were purchased from the
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Jackson Laboratory, Bar Harbor, Maine 04609 USA. Male C57BL/6J mice (20 to 25g) were obtained from Monash Animal Services, Monash University, Australia. α-SMA/EYFP (enhanced yellow fluorescent protein) mice were kindly provided by Dr. James Lessard (Cincinnati Children's Hospital Medical Centre, Cincinnati, Ohio, USA). In α-SMA/EYFP mice, EYFP expression is driven by the α-SMA promoter/enhancer, and is expressed not only in smooth muscle cells, but also in renal myofibroblasts. The isolation and culture of mouse renal CD31(+)EYFP(-) cells was described previously (10). All animal experiments were performed with the approval of a Monash University Animal Ethics Committee and adhered to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

The generation of RAGE-null mice and induction of diabetes in RAGE-null and wild type mice were described previously (13). Kidneys from 16 and 32 week old diabetic and non-diabetic mice were used for PAS staining and confocal microscopy analysis. Tie2-Cre;loxP-EGFP mice were generated by crossbreeding B6.Cg-Tg(Tek-cre)12F1v/J mice with B6.Cg-Tg(ACTB-Bgeo/GFP)21Lbe/J mice. For the time-course study, diabetes was induced in Tie2-Cre;loxP-EGFP mice (n=12) at 8 weeks of age by intraperitoneal administration of 50 μg/g STZ (Sigma-Aldrich, St. Louis, MO) for 5 consecutive days. Control Tie2-Cre;loxP-EGFP mice (n=6) received daily intraperitoneal injections of normal saline for 5 days. Mice were killed 1 and 3 month(s) after the onset of diabetes. Blood, urine and kidney tissue were analysed for biochemical parameters and renal histology. Urine in the bladder was collected for urinary albumin excretion once the mice were killed. The albumin-to-creatinine ratio was measured with Albuwell M and Creatinine Companion (Exocell, Philadelphia, PA).

A preliminary experiment was performed to determine the effective dose range of SIS3 in STZ-induced diabetic kidney disease. Diabetes was induced in male C57BL/6J mice by intraperitoneal injection of STZ as above. 1 month after the onset of diabetes, mice were divided into 4 groups (n=3) and given two intraperitoneal injections of 1, 2.5, 5μg/g SIS3 or vehicle with a 5hr interval and then killed 1hr after the second injection in order to examine the levels of renal Smad2 and Smad3 phosphorylation. Immunoprecipitation and western blotting demonstrated that 2.5μg/g SIS3 was sufficient to achieve more than 90% inhibition of phosphorylation of Smad3 compared with the vehicle-treated group without obvious side effects. Therefore, in the main study, to test the beneficial role of SIS3, 4 weeks after onset of diabetes mice were treated with the same volume of vehicle or 2.5mg/kg/d SIS3 delivered by implantation of an Alzet (Durect Corp., Cupertino, CA) osmotic pump for 8 weeks. By the experimental end point, mice were killed and blood, urine and kidney tissue were collected for analysis.

AGEs and MSA infusion study. AGEs (10μg/g/d, Sigma) or mouse serum albumin (MSA, 10μg/g/d, Sigma) was administered into male Tie2-Cre;loxP-EGFP mice (n=3) for 1 month by osmotic micropumps. To test whether SIS3 can block EndoMT in vivo, MSA (10μg/g/d), AGEs (10μg/g/d) + vehicle or AGEs (10μg/g/d) + SIS3 (2.5mg/kg/d) was administered into male Tie2-Cre;loxP-EGFP mice (n=3) for 1 month by separate osmotic micropumps. By the experimental end point, mice were killed and kidney tissues were collected for analysis.

Histology and confocal microscopy. The following antibodies were used for immunofluorescence studies: rat anti-CD31(BD Biosciences, San Diego, CA, USA); rabbit anti-Von Willebrand factor (vWF, Dako, Glostrup Denmark); mouse anti-α-SMA conjugated with Cyanine 3 (Cy3,
Sigma-Aldrich); rat anti-VE-cadherin (eBioscience, San Diego, CA, USA), rabbit anti-phosphorylated Smad3 (Novus Biologicals, LLC, Littleton, CO, USA), rabbit anti fibronectin (Sigma-Aldrich), goat anti-collagen IV, goat anti-rabbit Alexa Fluor 555 conjugate, goat anti-rat Alexa Fluor 647 conjugate and chicken anti-goat 647 conjugate (Invitrogen). Goat anti-RAGE neutralizing antibody and mouse anti-TGF-β1 neutralizing antibody (R&D Systems). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualise nuclei. Sections were analyzed with an Olympus Fluoview 1000 confocal microscope (Olympus, Tokyo), FV10-ASW software (version 1.7; Olympus), oil UPLFL 60x objective (NA 1.25; Olympus) at 2x, 3x or 6x digital zoom. Channels were acquired sequentially. Contrast and brightness of the images were further adjusted in Image J (rsbweb.nih.gov/ij). The degree of tubulointerstitial fibrosis was measured in 40 randomly selected high power fields (x600) in each animal using Image J software by analysing the percentage of the total cortical area accounted for by immunostaining for α-SMA, collagen IV or fibronectin. All scoring was performed blind on coded slides.

**Quantitation of myofibroblasts of endothelial cell origin.** EGFP(+)/α-SMA(+) cells were counted in renal cortex. Five cortical fields were analysed at x600 magnification in each of five sections from each kidney. The number of endothelial cell-origin myofibroblasts per mm² of cortex (excluding glomeruli) (EGFP(+)/α-SMA(+) cells/mm² of cortex) was determined, as well as the percentage of α-SMA(+)/EGFP(+) cells in total α-SMA(+) cells.

**MMEC culture.** MMECs were cultured as previously described (9). AGEs (Sigma-Aldrich Co.) were added at concentrations of 1, 5, 25 μg/ml to the cell cultures for 3 and 7 days in chamber-slides and 3, 6 and 24 hours in 6-well plates, with 25μg/ml BSA used as the control. In blocking studies, MMECs were pre-treated with goat anti-RAGE neutralizing antibody (4 μg/ml), mouse anti-TGFβ1 neutralizing antibody (4 μg/ml), normal goat IgG (4 μg/ml), normal mouse IgG (4 μg/ml), and SIS3 (2 μM, Sigma Alderich Co.) or vehicle (DMSO) for 30 mins then AGEs were added for different periods of time as described above. The treated cells were subsequently subjected to immunoprecipitation (IP)/western blotting (WB) and real-time PCR.

**RAGE, TGF-β-Receptor 1, Smad2 and Smad3 knockdown.** Control siRNA (Cat. No. 12935-200), RAGE siRNA (Cat. No. MSS218607), TGF-β receptor 1 siRNA (Cat. No. RSS355451), Smad2 siRNA (Cat. No. MSS206406) and Smad3 siRNA (Cat. No. MSS206422) were purchase from Invitrogen. For cell culture studies, mouse renal endothelial cells or MMECs were transfected in triplicate with RAGE siRNA, TGF-β receptor 1 siRNA, Smad2 siRNA and Smad3 siRNA or control siRNA using Lipofectamine 2000. Twenty-four hours following transfection, the transfected cells were subjected to BSA or AGEs stimulation for 30 mins, 24 hours or 48 hours. Cells were harvested for immunoblotting or real-time PCR analysis.

**RNA Extraction and Real-Time PCR.** Total RNA from kidneys or MMECs was isolated and RT-PCR and Real-time PCR performed with a RT-PCR kit (Invitrogen) and SYBR Green PCR Reagents (Sigma). Primers were as follows: mouse CD31, 5’-aggttcatagctcag and 5’-ctctgggttccagatgg; α-SMA, 5’-ctgacagggcccactgaa and 5’-gaaatagccaagctcag; mouse collagen IV 5’-aaagggagaaagggacttc and 5’-ctcttttgagcttcag; mouse fibronectin 5’-gaagaagcggcttcatgca and 5’-atctatcgggcatagca; mouse Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), 5’-cagatccacacgatatatg and
5'-catgacaaccttggcatgtgg. Reaction specificity was confirmed by electrophoretic analysis of products before real-time PCR and bands of expected size were detected. Ratios for CD31/GAPDH, α-SMA/GAPDH, collagen IV/GAPDH and fibronectin/GAPDH were calculated for each sample and expressed as the mean ± SD. The relative amounts of mRNA were calculated using the comparative Ct (ΔCt) method compared to GAPDH and expressed as the mean ± SD.

Immunoprecipitation and western blotting. Kidney and cell culture samples were sonicated and resuspended in 0.4 ml of RIPA lysis buffer. Protein concentration estimations were performed with a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Samples containing 500µg total protein were immunoprecipitated with a rabbit anti-Smad3 antibody (Cell Signalling Technology, Danvers, MA) followed by Western blotting with mouse anti-phosphoserine (Calbiochem, Kilsyth, Victoria, Australia) or mouse anti-Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-mouse IgM (Sigma-Aldrich) and bound antibody was detected by ECL Plus (Amersham, Little Chalfont, UK) and captured by Fujifilm model LAS-3000 (Fujifilm Corporation, Australia). Densitometry analysis was performed with Gel Pro analyzer (Media Cybernetics, Silver Spring, MD).

Statistical Analysis. Data are presented as mean ± SD with statistical analyses performed using one-way analysis of variance (ANOVA) from GraphPad Prism 5.0 or two-way ANOVA if appropriate (GraphPad Software, Inc., San Diego, CA, USA). Post-test Tukey's analysis was used when appropriate. A p value less than 0.05 was considered statistically significant.

RESULTS

AGEs induced EndoMT in MMECs and in Tie2-Cre;Loxp-EGFP mice. To investigate whether AGEs can induce EndoMT, we cultured MMECs in the presence of AGEs and unglycated BSA. MMECs have previously been shown to transdifferentiate into myofibroblasts in vitro upon TGF-β1 stimulation (10). Confocal microscopy (Fig. 1A-C) and real-time PCR (Fig. 1D-G) demonstrated that AGEs, but not BSA induced de novo expression of α-SMA, a putative marker of myofibroblasts. Concurrently, MMEC expression of the endothelial cell markers VE-cadherin (protein) and CD31 (mRNA) were lost in a time- and dose-dependent fashion. To corroborate the findings in vivo, AGEs or mouse serum albumin (MSA) were administrated to Tie2-Cre;Loxp-EGFP mice by osmotic micropumps. In Tie2-Cre;Loxp-EGFP mice, expression of EGFP in renal endothelial cells persists despite subsequent phenotypic changes (10). Confocal microscopy demonstrated that by 1 month after AGEs infusion, EGFP (+)/α-SMA (+) cells were present in the renal interstitium, but were not present in mice administered MSA (AGEs vs MSA, 5.4±1.4% vs 0.1±0.3%, p<0.05; Fig 2A-H and I). Thus, both in vitro and in vivo studies demonstrated the existence of AGE-induced EndoMT in microvascular endothelial cells.

RAGE-Smad3 crosstalk mediated AGE-induced EndoMT in MMECs. To investigate the expression of RAGE in renal endothelial cells, anti-RAGE and anti-CD31 antibodies were employed. Confocal microscopy demonstrated that the expression of RAGE was significantly increased in 1 month STZ-induced diabetic kidneys compared with normal saline-treated mouse kidneys (Fig 3A&B). The co-localized expression of RAGE with CD31 demonstrated the significant upregulation of RAGE expression in diabetic renal endothelial cells, both in glomeruli and in peritubular capillaries (Fig...
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To investigate whether AGE-induced EndoMT involves signalling through RAGE, RAGE siRNA was employed. Western blotting demonstrated that knockdown of RAGE abolished AGE-induced loss of VE-cadherin and de novo expression of α-SMA in mouse endothelial cells (Fig 3C). MMECs were also preincubated with goat anti-RAGE neutralizing antibody or goat IgG. Real-time PCR demonstrated that pre-treatment with the anti-RAGE neutralizing antibody, but not control goat IgG, reduced AGE-induced α-SMA mRNA expression by 64% and inhibited loss of CD31 by 60% compared with AGES-treatment alone and co-treatment with AGES and goat IgG (Fig 3D&E). To further corroborate RAGE mediated-EndoMT in diabetes, diabetes was induced in RAGE-null and wild type mice. PAS staining demonstrated that renal fibrosis was significantly reduced in RAGE-null diabetic kidney compared with RAGE-wild type diabetic kidney (Fig 4A-D). More importantly, confocal microscopy showed that the number of α-SMA(+) renal myofibroblasts and the percentage of vWF(+)/α-SMA(+) cells in total α-SMA(+) cells were significantly lower in RAGE-null mouse kidney than those in RAGE-wild type mouse kidney (Fig 4E-M). This suggests that AGE-induced EndoMT occurs largely through RAGE. Next we investigated whether AGES can induce Smad3 activation and whether AGE-induced Smad3 activation occurs through RAGE. Immunoprecipitation (IP)/western blotting (WB) demonstrated that incubation with AGES induced Smad3 activation in a time- and dose-dependent fashion in MMECs (Fig 5A&B). This AGE-induced Smad3 activation was reduced by 65% when MMECs were cultured in the presence of the anti-RAGE neutralizing antibody (Fig 5D). When RAGE siRNA was employed in MMECs, this AGE-induced rapid Smad3 phosphorylation was almost abrogated (Fig 5C). To further confirm RAGE-Smad3 cross-talk induced by AGES, an anti-TGF-β1 neutralizing antibody and TGF-β receptor 1 siRNA were employed in MMECs. Western blotting demonstrated that the anti-TGF-β1 neutralizing antibody could block TGF-β1-induced but not AGE-induced rapid Smad3 activation (30 mins) (Fig 5E). Western blotting also demonstrated that knockdown of TGF-β receptor 1 abrogated TGF-β1-induced but not AGE-induced rapid Smad3 activation (30 mins) in MMECs (Fig 5F). Thus, the experiment results have demonstrated that RAGE-Smad3 crosstalk is a major pathway mediating AGE-induced Smad3 activation in MMECs.

SIS3 abrogated AGE-induced Smad3 activation and EndoMT in MMECs. SIS3 has previously been shown to inhibit Smad3 phosphorylation in fibroblasts (28), and we reported that SIS3 abolished TGF-β1-induced EndoMT in MMECs (10). To investigate whether SIS3 can block AGE-induced activation of Smad3, MMECs were pretreated with 1μM SIS3 for 30 mins. IP/WB demonstrated that SIS3 abolished AGE-induced phosphorylation of Smad3, but not Smad2, suggesting the specificity of SIS3 in vitro (Fig 6A). Next we investigated whether SIS3 could inhibit AGE-induced EndoMT in MMECs. Confocal microscopy demonstrated that pre-treatment with SIS3 not only blocked AGE-induced de novo expression of α-SMA, but also inhibited AGE-induced loss of VE-cadherin in MMECs (Fig 6B-E), indicating the efficacy of SIS3 in inhibition of AGE-induced Smad3 phosphorylation and EndoMT in MMECs. To further differentiate the role of Smad2 and Smad3 in AGE-induced EndoMT, Smad2 siRNA and Smad3 siRNA were employed. Western blotting demonstrated that Smad2 siRNA and Smad3 siRNA significantly knocked down endogenous Smad2 and Smad3, respectively in MMECs (Fig 6F). Real-time PCR demonstrated that knockdown of Smad3 but not Smad2 almost abolished AGE-induced de...
novel expression of \(\alpha\)-SMA in MMECs (Fig 6G), suggesting the pivotal role of Smad3 in AGE-induced EndoMT.

**SIS3 inhibited Smad3 activation in STZ-induced DN in Tie2-Cre;Loxp-EGFP mice.**

Analysis of STZ-induced diabetic kidneys by IP/WB identified substantial Smad3 phosphorylation 1 month after induction of diabetes. Levels of Smad3 phosphorylation remained elevated 3 months after STZ administration (Fig 7A). Under multiple low-dose STZ injections regime, approximately 80% of the animals developed diabetes (29). IP/WB demonstrated that the level of Smad3 phosphorylation was not increased in STZ-treated non-diabetic mouse kidney compared with normal saline -treated and STZ-treated diabetic mouse kidneys (Fig 7B), suggesting that the elevated Smad3 phosphorylation is associated with diabetes, not STZ. Confocal microscopy further confirmed a significant increase in levels of phosphorylated Smad3 in renal endothelial cells of diabetic mice compared with normal saline-treated mice (Fig 7E-I). One month after the onset of STZ-induced diabetes, mice were given intraperitoneal injection of vehicle and different dosages of SIS3. IP/WB demonstrated that SIS3 blocked Smad3, but not Smad2 activation in STZ-induced diabetic mouse kidneys, suggesting the specificity of SIS3 in vivo (Fig 7C). To explore whether SIS3 can inhibit Smad3 activation in STZ-induced DN, SIS3 (2.5\(\mu\)g/g/day) or the same volume of vehicle was administered to Tie2-Cre;Loxp-EGFP mice by osmotic micropumps for 2 months, commencing 1 month after the administration of STZ. IP/WB demonstrated that after 2 months of SIS3 administration, Smad3 activation in STZ-diabetic mice was almost abolished compared with high levels of Smad3 activation in kidneys of vehicle-treated mice (Fig 7D). Confocal microscopy demonstrated that Smad3 activation in renal endothelial cells of STZ-diabetic mice was inhibited by SIS3 (Fig 7H-J), indicating the efficacy of SIS3 in vivo.

**SIS3 reduced AGE-induced EndoMT and decreased EndoMT in STZ-induced DN in Tie2-Cre;Loxp-EGFP mice.** To investigate the effect of SIS3 on AGE-induced EndoMT in vivo, MSA, AGEs+vehicle and AGEs+SIS3 were administered into Tie2-Cre;Loxp-EGFP mouse kidneys (Fig 8A-H). Next we examined the effects of SIS3 on EndoMT in STZ-induced DN in Tie2-Cre;Loxp-EGFP mice. Confocal microscopy demonstrated that SIS3 reduced the percentage of EGFP+/\(\alpha\)-SMA+ cells in total \(\alpha\)-SMA+ cells and the total number of \(\alpha\)-SMA+ cells in the renal interstitium compared with the vehicle-treated group (2.7\(\pm\)0.8% vs 14.2\(\pm\)4.0% and 30.3\(\pm\)10.5 cells/mm\(^2\) vs 158.7\(\pm\)91.1 cells/mm\(^2\), p<0.05, respectively, Fig 9A-N). This suggests that SIS3 not only inhibited EndoMT, but also reduced the accumulation of renal interstitial myofibroblasts.

The effects of SIS3 on the early development of renal fibrosis, macrophage infiltration and renal function. Confocal microscopy and real-time PCR demonstrated that compared with vehicle treatment, SIS3 significantly reduced collagen IV (Fig 10A-C, G&H) and fibronectin (Fig 10D-F, G&H) expression in the glomeruli and tubulointerstitium of STZ-treated Tie2-Cre;Loxp-EGFP mice. This suggests SIS3 retarded the early development of STZ-induced diabetic glomerulosclerosis and tubulointerstitial fibrosis. The renoprotective role of SIS3 was further confirmed by serum creatinine levels (STZ-DN+SIS3 vs STZ-DN+Vehicle, 0.088\(\pm\)0.013mg/dl vs 0.11\(\pm\)0.014mg/dl, p<0.05. Fig 10I). However, SIS3 administration did not reduce proteinuria (STZ-DN+SIS3 vs STZ-DN+Vehicle, Urine
DISCUSSION
The present study showed that AGEs can induce EndoMT in vitro and in vivo and also demonstrated the central role of the RAGE-Smad3 signalling pathway in AGE-induced EndoMT. More importantly, the present study demonstrated the efficacy of SIS3 in the inhibition of EndoMT in vitro and in vivo and the renoprotective effects of SIS3 in STZ-induced DN. Taken together, these findings suggest that EndoMT is a novel pathway leading to the development and progression of DN and that SIS3 has therapeutic potential for diabetic renal disease.

Zeisberg et al (9) and Li et al (10) have recently demonstrated that EndoMT mediates the pathogenesis of diabetic renal fibrosis. In addition to TGF-β1 and TGF-β2, the present study further identified AGEs as inducers of EndoMT. Given the increasing evidence demonstrating a causal role for AGEs in the development of diabetic complications, AGE-induced EndoMT may also be an important mechanism in the pathogenesis of diabetic retinopathy and vasculopathy. Thus, blockade of AGE-induced EndoMT may have therapeutic benefit in retarding the progression of diabetic complications.

Clinical trials of RAS inhibitors in patients with both type 1 and type 2 diabetes have shown protective effects on reducing renal and cardiovascular damage in patients with advanced DN with proteinuria (30, 31). However, large randomized clinical trials have revealed that inhibition of the RAS fails to prevent the development of early diabetic renal disease (5, 32), suggesting that additional mechanism(s) may participate in the development of diabetic renal disease. The present findings demonstrated that AGEs induced EndoMT in vitro and in vivo and that blockade of Smad3 phosphorylation by SIS3 significantly reduced EndoMT, decreased glomerulosclerosis and tubulointerstitial fibrosis and improved renal function. These findings suggest that EndoMT is a novel pathway leading to DN and that SIS3 may constitute a new measure to treat patients with DN.

SIS3 has been shown to inhibit Smad3 phosphorylation and abrogate TGF-β1-induced ECM production in fibroblasts (28). The present study demonstrated that SIS3 not only inhibited AGE-induced Smad3 activation and EndoMT in vitro, but also abrogated Smad3 activation, decreased EndoMT and retarded the progression of DN in vivo. Again, these findings suggest that blockade of Smad3 phosphorylation may have therapeutic potential for DN, although further studies are obviously required.

In addition to AGEs, other pro-fibrotic mediators, such as TGF-β and CTGF, are also elevated by AGEs in renal cells and can activate Smad3. Smad3 is activated in a variety of renal cells besides endothelial cells, such as tubular epithelial cells, fibroblasts and mesangial cells which also contribute to renal fibrosis. SIS3 inhibits Smad3 activation in these renal cells. Therefore the effect of SIS3 in the kidney is not only restricted to endothelial cells.

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Previous studies of Smad3 conditional knockout mice with STZ-induced diabetes
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(25, 26) or renal pathology due to UUO (27) have demonstrated the essential role of Smad3 in both glomerulosclerosis and tubulointerstitial fibrosis. However, the role of Smad3 in proteinuria remains controversial. Fujimoto et al (25) showed that compared with wild type mice, proteinuria was significantly decreased in Smad3 knockout mice with STZ-induced DN, while Wang et al (26) demonstrated that Smad3 deficiency limited diabetic glomerulosclerosis without affecting albuminuria. We have previously reported that EndoMT occurs and contributes to early renal interstitial fibrosis independently of microalbuminuria (10). In the present study, SIS3 treatment did not attenuate albuminuria. However, all of the above studies investigated proteinuria in the early stages of experimental diabetic kidney disease. The effect of SIS3 on proteinuria in advanced DN deserves investigation.

In the present study, the anti-RAGE neutralizing antibody and RAGE siRNA significantly inhibited AGE-induced EndoMT in both MMECs and mouse renal endothelial cells. Further, SIS3 almost completely blocked AGE-induced EndoMT in MMECs and in Tie2-Cre:Loxp-EGFP mouse kidneys. This suggests that RAGE is a major receptor involved in the induction of EndoMT while Smad3 plays an essential role in AGE-induced EndoMT. The administration of SIS3 to STZ-induced DN significantly reduced, but not completely retarded the development of diabetic renal fibrosis, suggesting that other pathological factors, such as activation of p38MAPK (33, 34), ERK (35, 36), and protein kinase C (37, 38), high glucose (39, 40), and oxidative stress (41, 42) may also be involved in the pathogenesis of DN.

In conclusion, the present study identified a novel mechanism in which AGE-induced EndoMT occurs and contributes to the development of diabetic renal fibrosis. RAGE-Smad3 crosstalk plays a central role in AGE-induced EndoMT. SIS3 not only inhibits EndoMT, but also prevents structural damage and provides renal functional protection. Blockade of EndoMT and RAGE-Smad3 crosstalk may provide a new strategy to retard the progression of DN and other diabetic complications.

Author contributions. J.L. researched data, wrote, reviewed and edited manuscript. X.Q. researched data. J.Y. researched data. G.C. reviewed/edited manuscript. S.R. contributed to discussion. Y.Y. researched data. H.Y. researched data. J.B. reviewed/edited manuscript.

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REFERENCES


FIGURE LEGENDS
Figure 1. AGEs induced EndoMT in MMECs. MMECs were cultured in the presence of AGEs (25μg/ml) or BSA (25μg/ml) for 3 and 7 days. Confocal microscopy demonstrated the expression of VE-cadherin (green), α-SMA (red) and DAPI nuclear staining (blue) in MMECs after culture with either BSA (A) or AGEs (B) for 7 days. Arrow indicates a VE-cadherin+/α-SMA+ cell. (C) Quantitation of the percentages of VE-cadherin- and α-SMA-positive cells in total DAPI-positive cells. One-way ANOVA, a, versus 7d BSA, p<0.05; b, versus 3d AGEs, p<0.05. Real-time PCR demonstrated mRNA levels of α-SMA (D&F) and CD31 (E&G) in MMECs cultured in the presence of BSA or AGEs for 3, 6 and 24 hrs (D&E), and with different concentrations of AGEs and BSA for 24 hrs (F&G). (D) Two-way ANOVA, time, p<0.05; treatment, p<0.05; interaction, p<0.05. (E) Two-way ANOVA, time, p>0.05; treatment, p<0.05; interaction, p<0.05. (F) c, versus 25μg/ml BSA or 1μg/ml AGEs, p<0.05; d, versus 5μg/ml AGEs, p<0.05. (G) e, versus 25μg/ml BSA or 1μg/ml AGEs, p<0.05; f, versus vs;5μg/ml AGEs, p<0.05.

Figure 2. Infusion of AGEs into Tie2-Cre;Loxp-EGFP mice induced EndoMT. Confocal microscopy demonstrated EGFP (green), α-SMA (red) and DAPI (blue) staining in MSA-treated (A) and AGEs-treated (B) mouse kidneys. Arrow indicates EGFP+/α-SMA+ cells in an AGEs-treated kidney—seen at higher magnification in C-E. Arrow heads indicate EGFP-/α-SMA+ cells in an AGEs-treated kidney—seen at higher magnification in F-H. Original magnification, A&B, 600x; C-H, 1200X. (I) Quantification of percentage of α-SMA+/EGFP+ cells in total α-SMA+ cells in MSA-treated and AGES-treated kidneys. a, versus MSA-treated group, p<0.05.
**Figure 3. AGE-induced EndoMT was RAGE-mediated in MMECs.** Confocal microscopy demonstrated the expression of RAGE (green), CD31 (red) and DAPI (blue) in kidney 1 month after administration of either normal saline (A) or STZ (B). Original magnification, A&B, 600x. (C) Mouse renal endothelial cells were treated with control siRNA (CTL siRNA) and RAGE siRNA for 24 hours then stimulated with BSA or AGEs for 48 hours. Western blotting demonstrated the expression of RAGE, VE-cadherin (VE-cad), α-SMA and GAPDH. (D&E) Real-time PCR demonstrated mRNA levels of α-SMA (D) and CD31 (E) in MMECs after culture with BSA, AGEs, AGEs + goat anti-RAGE neutralizing antibody or AGEs + goat IgG for 24 hrs. a, versus BSA, P<0.05; b, versus AGEs and AGEs + goat IgG respectively, P<0.05.

**Figure 4. RAGE-mediated EndoMT and diabetic renal fibrosis.** PAS staining showed histological changes in RAGE-wild type (RAGE+/+, A&C), RAGE-null (RAGE-/-, B&D), non-diabetic (A&B) and diabetic kidneys (C&D) at 32 weeks of age. Confocal microscopy demonstrated vWF (green), α-SMA (red) and DAPI (blue) in non-diabetic RAGE+/+ (E), non-diabetic RAGE-/- (F), diabetic RAGE+/+ (G-J), and diabetic RAGE-/- (K) kidneys. Original magnification, A-D, 400X; E-G&K, 600X; H-J, 1800X. Quantification of number of α-SMA(+) renal interstitial myofibroblasts (L) and percentage of vWF+/α-SMA+ cells in total α-SMA+ cells (M). a, versus non-diabetic kidneys, p<0.05; b, versus diabetic RAGE+/+ kidneys, p<0.05.

**Figure 5. AGE-induced activation of Smad3 was RAGE-mediated in MMECs.** MMECs were cultured in the presence of AGES and BSA for 15 to 120 mins or with different concentrations of AGES for 30 mins. Immunoprecipitation (IP) and western blotting (WB) demonstrated the time-course (A) and dose response (B) of Smad3 phosphorylation (p-Smad3) and total Smad3 levels in MMECs. (C) MMECs were pre-treated with control siRNA (CTL siRNA) or RAGE siRNA for 2 days then cultured in the presence of BSA or AGEs for 30 mins. IP/WB demonstrated RAGE, p-Smad3 and total Smad3 in MMECs. (D) MMECs were pre-treated with goat anti-RAGE neutralizing antibody or goat IgG for 30 mins then cultured in the presence of AGES. Upper panel: IP/WB demonstrated p-Smad3 and total Smad3 in MMECs. Lower panel: quantitation of arbitrary ratio of p-Smad3/Smad3 in 3 independent experiments. a, versus BSA-treated group, p<0.05; b, versus AGEs or AGEs+ goat IgG, p<0.05. (E) MMECs were pre-treated with mouse anti-TGF-β1 neutralizing antibody or mouse IgG for 30 mins then cultured in the presence of AGES for 30 mins. IP/WB demonstrated p-Smad3 and total Smad3 in MMECs. (F) MMECs were pre-treated with control siRNA (CTL siRNA) or TGF-β receptor 1 siRNA for 2 days then cultured in the presence of AGES for 30 mins. IP/WB demonstrated TGF-β receptor 1, GAPDH, p-Smad3 and total Smad3 in MMECs.

**Figure 6. SIS3 inhibited AGE-induced activation of Smad3 and EndoMT in MMECs.** MMECs were pre-treated with 1μM SIS3 or DMSO for 30 mins then cultured in the presence of 25μg/ml AGES for 30 mins or 7 days. IP/WB demonstrated p-Smad3, Smad3, p-Smad2 and Smad2 at 30 mins after AGES stimulation in the presence of SIS3 (A). Confocal microscopy demonstrated the expression of VE-Cadherin (green), α-SMA (red) and DAPI (blue) 7 days after incubation with BSA (C), AGES + DMSO (D) and AGES + SIS3 (E) in MMECs. Arrows indicate VE-Cadherin+/α-SMA+ cells. (B) Quantitation of percentages of α-SMA- and VE-cadherin-positive cells in total DAPI-positive cells. a, versus BSA-treated group or AGES + SIS3 −treated group, p<0.05. Original magnification, 600x. (F&G) MMECs were pre-treated with control siRNA (CTL siRNA), Smad2 siRNA or Smad3 siRNA for 2 days then cultured in the presence of AGES or BSA for 24 hrs. WB demonstrated Smad2, Smad3 and GAPDH in MMECs (F) and real-time PCR showed α-SMA mRNA level in MMECs (G). b, versus BSA, p<0.05. c, versus BSA, p<0.05.
Figure 7. SIS3 inhibited Smad3 activation in STZ-induced DN. IP/WB demonstrated (A) the time-course of phosphorylated Smad3 (p-Smad3) and total levels of Smad3 in STZ-induced diabetic mouse kidneys and normal saline (NS)-treated kidneys; (B) p-Smad3 and total Smad3 in NS-treated, STZ-treated non-diabetic (STZ+/DM-) and STZ-treated diabetic (STZ+/DM+) mouse kidneys. (C) One month after the onset of STZ-induced diabetes or normal saline (NS) treatment, diabetic mice were given intraperitoneal injection of vehicle and different dosages of SIS3. IP/WB demonstrated p-Smad3, total Smad3, p-Smad2 and total Smad2 in kidneys of mice with different treatments. (D) IP/WB demonstrated p-Smad3 and total Smad3 in NS-treated mouse kidneys, STZ-induced DN + vehicle-treated mouse kidneys and STZ-induced DN + SIS3-treated (2.5μg/g/d) mouse kidneys. Confocal microscopy demonstrated CD31 (green), p-Smad3 (red) and DAPI (blue) staining in 1 month NS-treated kidney (E), 1-month STZ-induced diabetic kidney (F), 3-month STZ-induced DN + vehicle-treated mouse kidney (H) and 3 month STZ-induced DN + SIS3-treated mouse kidney (I). (G) Quantitation of percentage of p-Smad3-positive cells in total CD31-positive cells in 1-month NS-treated and STZ-treated diabetic kidneys. (J) Quantitation of percentage of p-Smad3-positive cells in total CD31-positive cells in 3-month NS-treated, STZ-induced DN + vehicle-treated and STZ-induced DN + SIS3 treated kidneys. a, versus NS, p<0.05; b, versus STZ-induced DN + vehicle-treated kidneys, p<0.05. Original magnification, C, D, F & G, 600X.

Figure 8. SIS3 inhibited AGE-induced EndoMT in Tie2-Cre;Loxp-EGFP mice. Confocal microscopy demonstrated EGFP (green), α-SMA (red) and DAPI (blue) staining in MSA-treated (A), AGEs + vehicle-treated (B-F) and AGEs + SIS3-treated (G) mouse kidneys. Arrows indicate EGFP+/α-SMA+ cells in an AGEs + vehicle-treated kidney –seen at higher magnification in C-F. Original magnification, A, B&G, 600x; C-F, 1200X. Quantification of percentage of α-SMA+/EGFP+ cells in total α-SMA+ cells in MSA-treated, AGEs + vehicle-treated and AGEs + SIS3-treated mouse kidneys (H). a, versus MSA-treated group, p<0.05; b, versus AGEs + vehicle-treated group, p<0.05.

Figure 9. SIS3 reduced EndoMT in STZ-induced DN in Tie2-Cre;Loxp-EGFP mice. Confocal microscopy demonstrated EGFP (green), α-SMA (red) and DAPI (blue) in normal saline-treated kidney (A), STZ-induced DN+vehicle kidney (B-H) and STZ-induced DN+SIS3 mouse kidney (I). Arrows indicate EGFP+/α-SMA+ cells that are enlarged in C-E. (F), α-SMA (red); (G) EGFP (green); (H), DAPI (blue) and (B) merged. Confocal microscopy demonstrated α-SMA staining in normal saline (NS) –treated (J), STZ-induced DN+vehicle-treated (K) and STZ-induced DN+SIS3-treated (L) mouse kidneys. Quantitation of percentage of EGFP+/α-SMA+ cells in total α-SMA+ cells (M) and number of α-SMA+ myofibroblasts in NS, STZ-induced DN+Vehicle and STZ-induced DN+SIS3 mouse kidneys (N). a, versus NS, p<0.05; b, versus STZ-induced DN+vehicle, p<0.05. Original magnification, A, B, F-K, 600x; C-E, 1200X.

Figure 10. SIS3 reduces renal fibrosis in DN. Confocal microscopy demonstrated collagen IV (A-C) and fibronectin (D-F) immunostaining in normal saline (NS)-treated (NS, A&D), STZ-induced DN (STZ-DN)+vehicle (B&F) and STZ-induced DN (STZ-DN)+SIS3 (C&F) mouse kidneys. Original magnification, 600x. (G) Quantification of collagen IV and fibronectin staining in NS-treated, STZ-DN+vehicle-treated and STZ-DN+SIS3-treated kidneys. a, versus NS, p<0.05; b, versus STZ-DN+vehicle, p<0.05. (H) Real-time PCR demonstrated α-SMA, collagen IV and fibronectin mRNA expression in NS, STZ-DN+Vehicle, STZ-DN+SIS3 mouse kidneys.
Smad3 inhibition in diabetic renal fibrosis

c, versus NS, p<0.05; d, versus STZ-DN+vehicle, p<0.05. (I) Serum creatinine in NS, STZ-DN+vehicle and STZ-DN+SIS3 mouse kidneys. e, versus NS, p<0.05; f, versus STZ-DN+vehicle, p<0.05. (J) Urine albumin/creatinine in NS, STZ-DN+vehicle and STZ-DN+SIS3 groups. No significant differences were identified.

Figure 1
Figure 2

A  NS  B  STZ-DN + Vehicle  C  STZ-DN + SIS3

D  NS  E  STZ-DN + Vehicle  F  STZ-DN + SIS3

G

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Figure 3

A  EGFP  α-SMA  DAPI  B  EGFP  α-SMA  DAPI  C  EGFP  D  α-SMA  E  DAPI

F  EGFP  G  α-SMA  H  DAPI

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Smad3 inhibition in diabetic renal fibrosis

Figure 4

A. RAGE CD31 DAPI
B. RAGE CD31 DAPI
C. CTLsRNA RAGE/sRNA
BSA AGEs BSA AGEs
RAGE
VE-cad
α-SMA
GAPDH

D. 
BSA
AGES
AGES+RAGE Ab
AGES+Goat IgG

E. 
BSA
AGES
AGES+RAGE Ab
AGES+Goat IgG

Figure 5

A. Non-DM:RAGE(+)
B. Non-DM:RAGE(-)
C. DM:RAGE(+)
D. DM:RAGE(-)
E. vWFα-SMA DAPI
F. vWFα-SMA DAPI

G. vWFα-SMA DAPI
H. vWFα-SMA DAPI
I. Number of α-SMA+ cells

J. Number of α-SMA+ cells

K. Number of α-SMA+ cells

L. Number of α-SMA+ cells

M. Number of α-SMA+ cells
Smad3 inhibition in diabetic renal fibrosis

Figure 6
Smad3 inhibition in diabetic renal fibrosis

Figure 7

**A**

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**B**

% of marker+ cells in total DAPI+ cells

- BSA
- AGEs + Vehicle
- AGEs + SIS3

**C** VE-cadherin α-SMA DAPI

**D** VE-cadherin α-SMA DAPI

**E** VE-cadherin α-SMA DAPI

**F**

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**G**

α-SMA mRNA/GAPDH

- CTLsiRNA
- Smad2siRNA
- Smad3siRNA

- BSA
- AGES
- BSA
- AGES
- BSA
- AGES
Figure 8

A

B

C

D

E

F

G

H

I

J

Figure 9

A

B

C

D

E

F

G

H

egfP-α-SMA (DAPI)

egfP-α-SMA (DAPI)

egfP-α-SMA (DAPI)

% EGF+α-SMA in total eC31+ cells
Smad3 inhibition in diabetic renal fibrosis

Figure 10

A EGFP α-SMA DAPI
B EGFP α-SMA DAPI
C
D
E
F α-SMA
G EGFP
H DAPI
J α-SMA NS
K α-SMA STZ-DN+Vehicle
L α-SMA STZ-DN+SIS3

M % EGFP+α-SMA+ cells in total α-SMA+ cells

N Number of α-SMA+ myofibroblasts/mm²

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Significance:
a: P < 0.05 compared to NS
b: P < 0.05 compared to STZ-DN+vehicle