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Transforming Growth Factor β1-Mediated mRNA Expression And Oxidative Stress in Renal Mesangial Cells:Comparison With High Glucose And Hexosamine-Induced Gene Expression Profiles

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ABSTRACT

Recently, we demonstrated high glucose and glucosamine induced expression of thioredoxin interacting protein(TXNIP) and several genes involved in oxidative stress and endoplasmic reticular stress in mesangial cells in a microarray study. Transforming growth factor-beta(TGF-β) 1 is considered to be a mediator of renal hypertrophy and ECM gene expression in mesangial cells and the development of diabetic glomerulopathy. Here, we further investigate the effects of TGF-β1 on mRNA expression of a mouse mesangial cell line MES-13 using Affymetrix murine expression U430 2.0 Oligochips. The data obtained are further compared with the previously described high glucose (HG) and glucosamine (GlcN)-induced gene expression profile. We identified 264 genes (~0.7% of the total 34,000 genes present in the microarray) as TGF-β1-regulated genes at 1.5-fold differential expression of which 161 genes are up-regulated and 85-genes are down-regulated. Of the 264 genes, 63 were also targeted by HG and 38 genes by GlcN while 13 genes are commonly regulated by all three and others are unique to TGF-β1. Biological process-based ontological analysis of differentially expressed genes reveal that a majority of them are involved in (i) regulation of physiological processes, (ii) metabolism, (iii) transcription and DNA binding, (iv) cell cycle control and (v) differentiation. A number of genes identified in the microarray experiment are further validated by quantitative real time-PCR. TGF-β1 induces the expression of various cellular hypertrophic and fibrotic factors such as connective tissue growth factor, platelet-derived growth factor B and fibroblast growth factor 21. Furthermore, TGF-β1 increases reactive oxygen species (ROS) generation, TXNIP expression and apoptosis of mesangial cells as revealed by TUNEL. Extracellular matrix related genes targeted by TGF-β1 are osteopontin, tenascin C, fibronectin, laminin β3, plaminogen activator inhibitor (PAI)-1 and cell surface glycoprotein modifying I-type antigen enzyme, glucosaminyl (N-acetyl) transferase 2. There also exist cross-talks between TGF-β1 and IFN-γ signaling pathways as revealed by cis-acting reporter activity assay for STAT3, γ-activated sequence (GAS) and interferon response sequence element (ISRE). The common targets of HG, GlcN and TGF-β1 induced genes include TXNIP, osteopontin, PAI-1 and others. The results reveal new signaling pathways and gene expression patterns that were not previously understood and reinforces the hypothesis that TGF-β1 mediates several harmful effects of chronic hyperglycemia and hexosamines to generate ROS, ECM accumulation and apoptosis of renal mesangial cells in diabetic glomerulopathy.

KEYWORDS

Transforming growth factor-β1; Mesangial cell; Transcriptome profiling; ECM gene expression; Oxidative stress; TXNIP and apoptosis.

INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal disease and characterized by renal hypertrophy, glomerular and tubular basement thickening, and mesangial matrix expansion with extracellular matrix(ECM) protein accumulation[37,47]. High concentrations of glucose, various cytokines and growth factors contribute to the accumulation of ECM proteins in the kidney during diabetes[40]. Prominent among these factors is the cytokine transforming growth factor-β1(TGF-β1) that has been proposed as one of the key mediators of ECM accumulation in the development of human and experimental diabetic kidney. TGF-β1 is a polypeptide growth factor thought to play an important role in a large spectrum of cellular processes such as early embryonic development, cell growth, differentiation, motility and apoptosis. In mesangial cells, the increases in TGF-β1 and fibronectin production are mediated by an activation of the promoters of the corresponding genes. Promoters of the TGF-β1[15] and the fibronectin gene[24] are stimulated by high glucose, and TGF-β1 also activates the fibronectin promoter[17]. Transcription factors of the activating protein 1(AP-1) family and cyclin AMP resposive element(CRE) binding protein(CREB), GC binding factor Sp1 and others are known to be activated by high glucose in mesangial cells[24,48] and mediates the transcriptional activation of the TGF-β1 and ECM genes[26,41]. TGF-β1 functions through activation of multiple intracellular signal mechanisms such as the Smad family of proteins[9,34], mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinases(ERKs), the c-jun N-terminal kinases, and the p38MAPK[12] leading to the increased expression of ECM proteins, protease inhibitor plasminogen activator inhibitor(PAI)-1, and other ECM-degrading proteases[35,39]. However, the global effects of TGF-β1 on gene expression and signal transduction in renal mesangial are not fully understood as yet.

We have previously shown that several effects of excess glucose on mesangial ECM synthesis occur via the hexosamine biosynthesis pathway(HBP) and TGF-β1 expression[7,19,42,43]. Glucose 6-phosphate (Glc-6P) enters the HBP following conversion to fructose 6-phosphate(Fru-6P) and then to glucosamine 6-phosphate(GlcN-6P) by the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Subsequently, GlcN-6P, is acetylated and uridinylated(UDP-GlcNAc) to serve as substrates for glycoprotein/glycolipid synthesis[42]. We also reported that TGF-β1 plays an important role not only in the regulation of ECM gene expression but also in protein N-linked glycosylation and apoptosis of mesangial cells[19]. Furthermore, our recent analysis of high glucose and glucosamine mediated gene expression profile of mesangial cells reveals several important features of hyperglyemica-induced regulation of thioredoxin interacting protein(TXNIP) overexpression, an inhibitor of thioredoxin activity and inducer of oxidative stress, ECM expression and apoptosis of renal mesangial cells via the HBP flux[5]. In this study, we further performed a similar transcriptomic profile screening of mesangial cells to investigate the TGF-β1-mediated mRNA expression patterns using the Affymetrix murine expression U430 2.0 high densitiy chips. The results were further compared with the high glucose and glucosamine-induced transcriptome profile. We observed that mesangial cells express ~26126 genes(~58%) of the total 34,000 genes present in the microarray and have identified 246 genes to be differentially regulated by TGF-β1 at 1.5-folds change(161 genes up and 85 genes down). A significant number of genes, which are known to be involved in cell cycle arrest, oxidative stress and ECM regulation, are targeted by TGF-β1 in mesangial cells, and these genes are also similarly regulated by high glucose and hexosamine. There exist cross-talks between TGF-β1 and IFN-γ signaling mechanisms involving Stat3, gamma activated sequence(GAS) and interferon response sequence element(IRSE) dependent pathways. Additional growth factors and genes previously unknown to be regulated by TGF-β1 are identified in this study, which may play an important role in the development and progression of diabetic kidney disease.

EXPERIMENTAL

Cell culture

Stable murine mesangial(MES-13) cells transformed with non-capsid-forming SV-40 virus were

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obtained from the ATCC, Manassas, VA. They were maintained in DMEM and F-12 nutrient mixture (Ham's) (4:1 ratio) (GIBCO BRL, Gaithersburg, MD) containing a normal D-glucose concentration of 5.5mmol/L, 2% FCS, 100µg/ml streptomycin, 100U/ml penicillin, and 2 mmol/L glutamine[19]. The cells were incubated in a humidified incubator of 5% CO_2 at 37^{0}C and routinely passaged at confluence every 3 days by trypsinization using 10cm culture dishes. Approximately 70% confluent monolayers were starved in the above medium without FCS for 1 day and then incubated in freshly prepared minus serum medium with or without 10ng/ml TGF-β1 for 24 hours.

Total RNA isolation, cDNA and cRNA synthesis and genechip hybridization

RNA samples were prepared from a pool of three different culture dishes for each condition of Control(without TGF-β1) or with TGF-β1(10ng/ml, 24h) in order to minimize biological and technical variability. Total RNA was isolated using trizol reagent(Life technologies, inc., massachusetts). cDNA and cRNA synthesis, genechip hybridization, and scanning of the affymetrix murine expression U430 2.0 chips(the affymetrix M430 2.0 chip contains 39,000 transcripts targeted at 34,000 well characterized genes) were performed according to the manufacturer's proto-col(Affymetrix, santa clara, CA) as recently described[5].

Microarray data analysis

The chips were read with affymetrix GCOS v1.2, and the probe intensity files were modeled with DChip 1.3(Harvard school of public health) in both PM-only and PM-MM modes as described before[5]. Consensus differentially regulated genes were initially derived from repeat experiments on the bases of a 90% CI of greater than 1.5-fold change in expression and with $p<0.05$ of error in paired t-test across repeats. This was further validated through modeling of variance between sample groups(one-way ANOVA) conducted in GeneSpring 6.0(Silicon genetix). The consensus gene-set was clustered in DChip and genespring 6.0, with ontoexpress(Wayne state university) to explore ontological associations. Further ontological and pathway analyses were conducted with the genego software and NIH's DAVID bioinformatics programs(http://appls.niaid.nih.gov/david).

Real-time quantitative PCR

Gene expression data from microarray analysis were confirmed by real-time quantitative PCR using the ABI Prism 7900HT sequence detection system and SYBR Green PCR master mix from applied biosystems. primers were designed using primer express v 2.0(Applied biosystems) and synthesized by invitrogen. The primers of 12 representative genes used in this study(TABLE 1). The real time PCR reaction system contained 1X SYBR green PCR Master Mix, 400nM forward and reverse primers, and 10 ng total cDNA in a volume of 25µl. The RT-PCR cycling was programmed as 95^{0}C for 15s and 60^{0}C for 1 min for 45 cycles followed by the construction of a melting curve through increasing the temperature from 60^{0}C to 95^{0}C at a ramp rate of 2% for 20min as described[5].

Promoter-enhancer-mediated gene expression pathway analysis:

Luciferase-containing reporter plasmids of pSTAT3-TA-Luc, pGAS-TA-Luc, and pISRE-TA-Luc with a common TATA box(TA) were used for interferon gamma pathway profiling as recently described in our laboratory[5]. The plasmid DNA was transfected into mouse MES-13 cells using Lipofectamine^{TM} 2000 kit(Invitrogen). The luciferase activities were measured, after treatments of the cells with or without 10 ng/ml TGF-β1 for 24 h, using the luciferase reporter assay kit(Promega, madison).

Intracellular reactive oxygen species(ROS) measurement

The formation of intracellular ROS in MES-13 cells was detected by using the fluorescent probe, 5-and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester(CM-H_2DCFDA) (Molecular probes inc.) as described previously(Singh et al. 2006). Brielfy, MES-13 cells were exposed to TGF-β1(10ng/ml) for 0-48 h. Approximately 1×10^5 cells/ml were cultured in 12 well plates, serum-starved overnight and TGF-β1 was added for 24h. Then, CM-H_2DCFDA(10µM) was incubated for 60min at 37^{0}C, and the fluorescence was measured in a gemini microplate reader fluorimeter with the bottom read scanning mode at 480nm excitation and emission at

TABLE 1: Oligonucleotide primers used to validate the micro array data

	Primers	Gene, Accession number and sequence (5'->3')	Region	Amplicon size
	ATF3	Activating transcription factor 3, BC019946		
1	forward	GAGGATTTTGCTAACCTGACACC	94-116	110
	reverse	TTGACGGTAACTGACTCCAGC	203-183	
	CTGF	Connective tissue growth factor; NM_010217		
2	forward	GGGCCTCTTCTGCGATTTC	231-249	151
	reverse	ATCCAGGCAAGTGCATTGGTA	381-361	
	P21Cip1	Cyclin-dependent kinase inhibitor 1A (P21); AK007630		
3	forward	CCTGGTGATGTCCGACCTG	10-28	103
	reverse	CCATGAGCGCATCGCAATC	112-94	
	FGF21	Fibroblast growth factor 21; NM_020013		
4	forward	CTGCTGGGGGTCTACCAAG	67-85	154
	reverse	CTGCGCCTACCACTGTTCC	220-202	
	GlcN-T	O-linked N-acetylglucosamine transferase; AF363030		
5	forward	AGGAAATGTCTTGAAAGAGGCAC	4-24	120
	reverse	TCGTAGTACACACAAGCCAGG	132-112	
	LAMB3	Laminin, beta 3, NM_008484		
6	forward	GGCTGCCTCGAAATTACAACA	215-235	220
	reverse	ACCCTCCATGTCTTGCCAAAG	434-414	
	PAI-1	Serine (or cysteine) proteinase inhibitor E1; NM_008871		
7	forward	TTCAGCCCTTGCTTGCCTC	12-30	116
	reverse	ACACTTTTACTCCGAAGTCGGT	127-106	
	TLR2	Toll-like receptor 2; NM_011905		
8	forward	GCAAACGCTGTTCTGCTCAG	56-75	231
	reverse	AGGCGTCTCCCTCTATTGTATT	286-265	
	KLF4	Kruppel-like factor 4 (gut); BG069413		
9	forward	GTGCCCCGACTAACCGTTG	86-104	185
	reverse	GTCGTTGAACTCCTCGGTCT	270-251	
	KLF5	Kruppel-like factor 5; BG069607		
10	forward	CCGGAGACGATCTGAAACACG	140-160	233
	reverse	GTTGATGCTGTAAGGTATGCCT	372-351	
	TXNIP	Thioredoxin interacting protein AF173681		
11	forward	TCTTTTGAGGTGGTCTTCAACG	25-46	104
	reverse	GCTTTGACTCGGGTAACTTCACA	128-106	
	TIEG1	TGF-β inducible early growth response 1 NM_013692		
12	forward	GTGACCGTCGGTTTATGAGGA	1298-1318	70
	reverse	AGCTTCTTGGCTGATAGGTGG	1367-1347	
	S18	Ribosomal S18 RNA; NM_138946		
13	forward	GCACAGTGTTTGTAGAGCCTG	24-44	147
	reverse	GCCCTGGAACTTATTGATCGGG	170-149	

530nm. After the measurement, the solution was aspirated and protein contents were determined with the biorad protein assay reagents.

TUNEL apoptosis detection assay

Apoptotic mesangial cells were detected by a

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terminal deoxynucleotidyl transferase biotin-dUTP end labeling(TUNEL) kit(Upstate cell signaling solutions, lake placid, NY) in which biotinylated dUTP is transferred to the free 3′OH of cleaved DNA by terminal deoxynucleotidyl transferase(TdT). MES-13 cells were grown under conditions similar to those described above for ROS determination. Cells were fixed with 4% paraformaldehyde in 0.1M NaH_2PO_4, pH 7.4 for 15 minutes at room temperature and permeabilized by 0.05% Tween-20 in 0.2% BSA in PBS for 15 minutes. Then, the cells were incubated with the fluorescein-labeled TUNEL reaction mixture for 1h at room temperature. Negative controls were obtained by omitting TdT enzyme and positive controls were performed with 2-5μg/ml DNase I in PBS for 1h at 37°C. After washing the cells with PBS three times, the coverslips were mounted with an aqueous based anti-fade medium for observation under the fluorescent microscope fitted with a DP70 digital camera for image acquisition.

SDS-PAGE and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis of proteins were performed as previously described[20]. The primary antibodies for Ser 133 phosphorylated and non-phosphorylated CREB were purchased form cell signaling technologies(Danvers, MA). Antibodies for laminin β1, FGF-21, thioredoxin(TRX) and Sp1 were obtained from Santa Cruz, CA. TXNIP antibodies were purchased from MBL International (Woburn, MA) and Ser/Thr-O-GlcNAc specific antibody RL2 was obtained from Affinity biotechnology(Golden, CO). They were used at a 1:1000 dilution in Tris-buffered saline pH 7.4 containing 5% non-fat dry milk and HRP-conjugated secondary antibodies was 1:3000. ECL(Amersham) was used to detect the immunoreactive bands. Wheat germ agglutinin binding was performed as described previously[19].

Immunofluorescence microscopy

Mesangial cells were grown in four-chambered tissue culture glass slides and exposed TGF-β1 for 0, 4, 24h. Cells were fixed in freshly prepared 4% paraformaldehyde, and permeabilzed with 0.2% Triton X. The slides were incubated with 5% normal horse serum for 30 minutes to block non-specific antibody binding, and after washing with PBS, cells were treated with WGA-RITC directly or with primary chicken anti-fibonectin or α-tubulin at 1:100 dilutions for both antibodies for 1.5h at room temperature. After washing with unbound primary antibodies, Alexa 488(for fibronectin) or Alexa 544-labeled appropriate secondary antibodies(Molecular probes) were added at 1:500 dilution for 1h at room temperature in a darkened chamber. After washing with PBS, the culture slides were mounted with coverslips using anti-fade media and shield with nail-polish. The cell-associated fluorescence was observed under an olympus BX51 fluorescent microscope, which is fitted with a triple DAPI/FITC/TRITC cube, a DP70 digital camera and image acquisition software. Images were captured either in single channel or double channel for Alexa fluor 488 and 544 and analyzed by the image analysis software of the olympus BX51.

Induction of diabetes in rats

Diabetes was induced in male wistar rats(200-220g) by intraperitoneal injection of streptozotocin (STZ) (55mg/kg body weight) as previously described[20,23]. After 2 months, they were killed and the kidneys were removed. The renal cortex was dissected out and stored immediately at -80°C until used. Treatment of animals conformed to the NIH Principles of laboratory animal care, and the WSU guidelines.

Statistical analysis

Results from real time PCR, promoter reporter assay and reactive oxygen species measurement are expressed as means +/-SE of indicated number of experiments. Student's t test is used to compare differences between cultures. A value of $p<0.05$ is considered statistically significant.

Gene lists and deposition

Four lists of genes that showed up-regulation and down-regulation upon TGF-β1 treatment of mouse MES-13 cells were summarized and deposited via the publicly available gene ontology data bases(web site: http://www.ncbi.nih.gov/projects/geo/query/acc.cgi?acc=GSE2558).

RESULTS

TGFβ1-regulated transcriptome patterns in mouse MES-13 cells

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The global gene expression profiles of TGF β1-treated and control mouse MES-13 cells were compared using affymetrix murine expression U430 2.0 chips. This oligo-DNA chip contains 45,100 probe sets equivalent to 39,000 transcripts or 34,000 genes, of which each probe set contains 11 perfect matched and 11 mismatched oligonucleotides(25 mer). Twenty four hour treatment of mouse mesangial cells with10 ng/ml TGF-β1 resulted in significant changes of transcriptome patterns as compared to untreated cells. Of the total 34,000 genes, 26126(~58%) genes were detected on the chip. A total number of 161 genes were up-regulated(see supplementary data TABLE 1S) and 85 genes were down-regulated (Supplementary TABLE 2S) by TGF-β1 using a 1.5 fold change bidirectionally as a cut-off threshold in two repeats of oligochip microarrays. The higher levels of up-regulated gene expression by TGF-β1 treatment are 4.5-fold increase(ranging from 4.22~4.78 folds) and 5.01-fold increase(ranging from 4.42~5.60 folds), which are a serine(or cysteine) proteinase inhibitor, clade E member 1 gene(PAI-1) and a cathepsin W gene, respectively. The down-regulated gene expression by TGF-β1 treatment at the minimum level are approximately -3.08, -3.40, and -3.60 folds respectively, for sclerostin domain containing 1, thioredoxin interacting protein and inhibitor of DNA binding 4 gene. The increased expression of connective tissue growth factor(CTGF, 2.18+/-0.21), fibroblast growth factor 21(FGF-21, 4.54+/-0.72) and platelet-derived growth factor polypeptide B(PDGF-B, ~2.01+/-0.15) by TGF-β1 is also seen in MES-13 cells. TGF-β1-regulated genes were further clustered by biological processes and metabolic function(TABLE 2) and most (i) up-regulated genes are involved in the regulation of cellular physiological processes, metabolism, cell cycle progression, cell adhesion and DNA and protein binding and transcriptional activities, while the (ii) down-regulated genes are involved in biological processes such as catalytic, transferase or lipid binding activities.

Real-time QPCR validation of microarray data

To further confirm differential gene expression in mesangial cells after TGF-β1 treatment, we analyzed several genes using real-time PCR. The results demonstrate that the gene expression direction and level match well between the microarray and real-time PCR methods(Figure 1 & TABLE 1 for primer sets), confirming the DNA microarray data even though real-time QPCR is a more sensitive method for gene expression analysis. For most genes tested in this study, the relative expression falls within 1.5-2.0 fold changes between the microarray data and real-time PCR except for FGF-21, which is ~4.5 fold increase by microarray but ~12 fold increase by real-time QPCR. Similarly, PAI-1 expression, an important regulator of ECM degradation, is also slightly higher in the Q-PCR when compared with the microarray data. The relative expression of thioredoxin inhibitor, TXNIP mRNA upon TGF-β1 treatment is decreased by 3.4+/-0.23 folds in microarray and 4.8+/-0.16 folds by real-time QPCR method. TGF-β inducible early growth response 1(TIEG1) and $P21^{Cip1}$ are also increased and they may involve in cell cycle

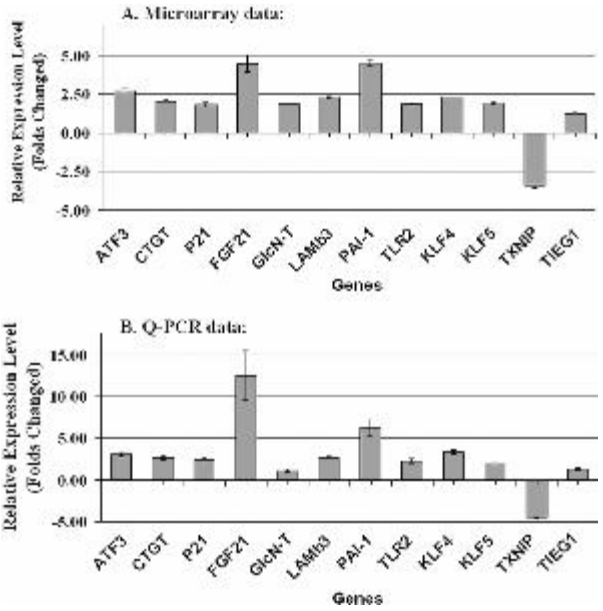

Figure 1: Validation of microarray results by real-time Q-PCR

MES-13 cells were incubated in control (NG, 5.5 mM) or with TGF-β1 (10 ng/ml) for 24 h. Total RNA was isolated using trizol reagent and performed the real-time quantitative PCR as described in Methods. (A) Quantitative analysis of the expression of some representative genes detected by microarray, and (B) the expression of the genes detected by real-time Q-PCR are presented. The data are analyzed from two repeats in microarray and from three repeats of real-time PCR. The relative expression level of each gene is presented here as the average +/- SE against the control. Ribosomal RNA 18S is used to normalize the mRNA level of each gene. The real-time Q-PCR is found to be more sensitive than the microarray data. The names of genes, their abbreviations and primer sets are listed in TABLE 1

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TABLE 2: TGF-β1 regulation of biological processes in mouse mesangial cells

Term	Count	%	p Value
Regulation of biological process	31	32.6%	1.49E-6
Regulation of physiological process	27	28.4%	1.71E-6
Regulation of metabolism	24	25.3%	4.65E-5
Regulation of transcription, dna-dependent	22	23.2%	9.74E-5
Transcription, dna-dependent	22	23.2%	1.32E-4
Regulation of transcription	22	23.2%	1.50E-4
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	22	23.2%	1.59E-4
Regulation of cell cycle	9	9.5%	2.27E-4
Transcription	22	23.2%	2.70E-4
Morphogenesis	14	14.7%	1.79E-3
Organogenesis	12	12.6%	4.28E-3
Cell cycle	10	10.5%	4.43E-3
Cell differentiation	7	7.4%	5.97E-3
Cell proliferation	11	11.6%	7.4E-3
Development	16	16.8%	1.28E-2
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	23	24.2%	1.42E-2
Positive regulation of transcription from pol ii promoter	3	3.2%	1.49E-2
Organismal physiological process	15	15.8%	1.62E-2
Positive regulation of metabolism	4	4.2%	1.85E-2
Protein amino acid dephosphorylation	4	4.2%	2.52E-2
Positive regulation of transcription, dna-dependent	3	3.2%	3.35E-2
Phosphate metabolism	9	9.5%	4.2E-2
Phosphorus metabolism	9	9.5%	4.2E-2
Regulation of transcription from pol ii promoter	4	4.2%	4.28E-2
Negative regulation of apoptosis	3	3.2%	6.17E-2
Negative regulation of programmed cell death	3	3.2%	6.17E-2
Positive regulation of transcription	3	3.2%	6.30E-2
Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	3	3.2%	6.44E-2
Cell adhesion	7	7.4%	7.97E-2
Cellular physiological process	28	29.5%	8.11E-2
Cell motility	4	4.2%	9.27E-2

TGF-β1-induced differentially expressed genes versus control in MES-13 cells were analyzed and categorized for cellular functions by using the KEGG biochemical pathway database as described in Methods

arrest and hypertrophy of renal mesangial cells and the diabetic kidney. We have previously shown that TIEG1 is stimulated by high glucose and glucosamine at 2-folds[5] and TGF-β1 stimulates it at 1.44-folds in the present microarray analysis and ~1.65-folds by QPCR. TIEG1 is known to play an important role in the up-regulation of PAI-1 and suppression of Smad 7 transcription, an inhibitor of Smad 3/4 signaling pathway.

TGF-β1 mediated ECM gene expression in mesangial cells

We have previously demonstrated that high glucose and glucosamine increase TGF-β1 promoter activity in mesangial cell[7] and TGF-β1 uses similar signaling pathways as hexosamines to mediate matrix protein synthesis in mesangial cells[43]. Our present study provided a set of TGF-β1-induced extracellular matrix gene expression in mesangial cells shown in TABLE 2. Approximately twenty eight of 47 ECM related genes identified on oligochip microarray are up-regulated by TGF-β1. These include fibronectin 1 and 3, laminin β3, laminin γ2, integrin binding

sialoprotein, procollagen isotypes, FBJ osteosarcoma oncogenes, tenascin C and PAI-1. TGF-β1 also increases the expression of cell surface glycoprotein and glycolipid I-type antigen modifying enzyme, β1-6 lactosamine N-Acetyl glucosamine transferase 2(GlcN-T2), which correlates with increased wheat germ agglutinin binding, a GlcNAc specific lectin, to mesangial cells and elevated level of fibronectin after treatment of the cells with TGF-β1 for 4 and 24 h(Figure 2A).

Mesangial cells are known to expression laminin 8 and 9 comprising of α4, laminin β1, β2 and γ1; however, their mRNA levels are not altered significantly by TGF-β1 in this study although the proteins accumulate in the diabetic kidney[19,43]. Therefore, increased glycosylation of matrix proteins by GlcN-T2 expression and/or decreased protein degradation via up-regulation of PAI-1 by TGF-β1 will definitely influence the overall ECM accumulation in the diabetic kidney. In concert with this hypothesis, we observed that the level of laminin β1 protein is increased by TGF-β1 in mesangial cells on western blots(Figure 2B), and that tunicamycin, an inhibitor of N-linked protein glycosylation in cells, inhibits laminin β1 binding to WGA-agarose(Figure 2C). TGF-β1 retards the mobility of laminin β1 protein in gels similar to those observed previously for laminin γ1 subunit due to increased protein glycosylation[19]. Furthermore, TGF-β1 induces the expression of laminin β3 and laminin γ2, which are components of endothelial cell associated laminin 5(α3β3γ2) (TABLE 3).

Signaling pathways involved in TGF-β1 function

Identification of the specific regulatory steps in TGF-β1 signaling is important to elucidate the TGF-β1 mediated signal mechanisms in renal mesangial cells. Both DNA microarray-based bioinformatics and cis-regulatory element-reporter gene assay methods were employed to study the potential TGF-β1 signaling pathways in mouse MES-13 cells. Several signaling pathways were significantly regulated by TGF-β1 treatment in mouse MES-13 cells. They are 1. ANG II(AngII, C-JUN, C-FOS); 2. IFN(IFN-γ inducible gene 203, IFN-γ inducible gene 205, interferon-induced protein with tetratricopeptide repeats 3); 3. ERK(C-MYC, C-JUN, C-FOS); 4. Glucocorticoid receptor(C-JUN, C-FOS, PAI-1); and 5. hedgehog (RUNX2, C-FOS, osteopontin). Additional signaling pathways regulated by TGF-β1 are the HBP(GlcN-T2, hexokinase 2) and toll like receptor 2. The ERK signaling pathway has been shown to enhance reactive oxygen species(ROS) generation, oxidative stress and glomerular injury[16].

Interferon inducible genes are targets of high glucose and glucosamine in mouse mesangial cells[5] and they are also up-regulated by TGF-β1 in this study. Therefore, we investigated the induction of cis-regu-

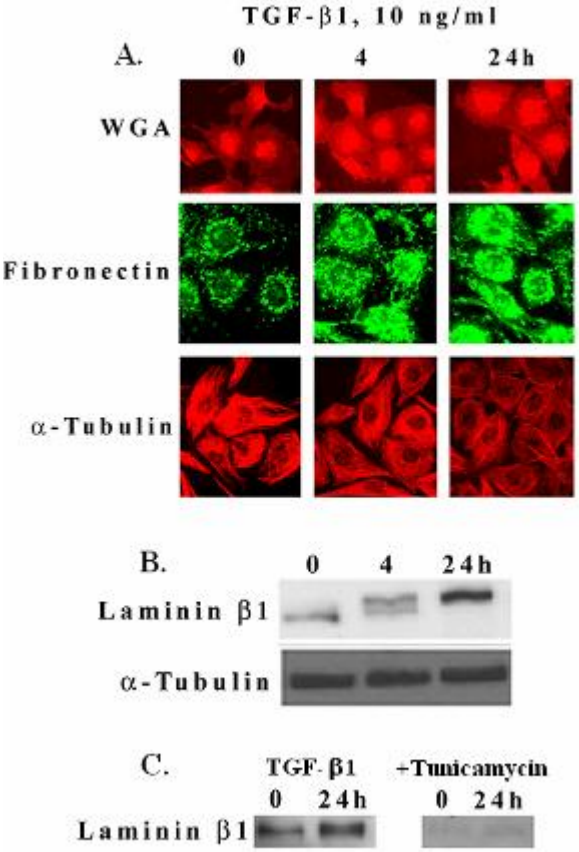

Figure 2: TGF-β1 increases cell surface glycosylation and ECM expression in mesangial cells

MES-13 cells were incubated with or without TGF-β1 for 0, 4, or 24 h in serum-starved medium either in four-chamber slides for immunohistology or in 100 mm culture dishes for western blots as described in methods. (A). Immunohistology. Wheat germ agglutinin-RITC labeling (upper panel), fibronectin-alexa 488 (middle) and α-tubulin-Alexa 544 (lower panel). (B). Western blot. Total cell extracts (30 μg) were loaded on to SDS-PAGE and probed with anti-laminin β1 on western blots. Membranes were reprobed for α-tubulin. A representative blot of n=4 is shown here. C. WGA-agarose binding. MES cells were cultured with or without TGF-β1 for 24 in the presence or absence of tunicamycin 2.5 μg/ml to block N-linked protein glycosylation. Cellular glycoproteins were separated on WGA-agarose columns and eluted by 0.25 M N-acetyleglucosamine and subjected to western blotting. TGF-1 increases laminin β1 binding to WGA-agarose and tunicamycin treatment blocks the binding. A reprentative of n=3 is shown here

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TABLE 3: TGF-β1 induction of ECM related genes in renal mesangial cells

Sl #	Gene	Accession	Average	se
1	FBJ osteosarcoma oncogene	AV026617	1.72	0.01
2	FBJ osteosarcoma oncogene B	NM_008036	1.6	0.02
3	Fibronectin 1	BC004724	1.78	0.01
4	Fibronectin 1	BM234360	2.18	0.16
5	Fibronectin 3	AI850911	1.67	0.38
6	FN3 domain containing 3	BC022140	1.14	0.02
7	Integrin binding sialoprotein	L20232	1.73	0.42
8	Intergrin α5	AI481717	1.19	0.15
9	Intergrin β1	BM120341	1.34	0.18
10	Intergrin β1	BB443308	1.25	0.17
11	Laminin β1	BG970109	1.23	0.06
12	Laminin β3	NM_008484	2.31	0.04
13	Laminin γ2	NM_008485	1.34	0.05
14	Matrix metalloproteinase 1α	NM_032006	1.27	0.22
15	Matrix metalloproteinase 1β	NM_032007	1.21	0.05
16	Osteoclast inhibitory lectin related protein	NM_027562	1.23	0.16
17	Osteoglycin	BB542051	1.26	0.08
18	Osteomodulin	NM_012050	1.29	0.24
19	Procollagen I, α1	BI794771	1.55	0.13
20	Procollagen IV, α3	AV366831	1.47	0.07
21	Procollagen IV, α4	AF169388	1.55	0.15
22	Procollagen IX. α3	AV291496	1.27	0.11
23	Procollagen lysine	BC0213521	1.31	0.02
24	Procollagen V, α1	AW744319	1.51	0.05
25	Procollagen VII, α1	NM_007738	1.26	0.02
26	Secreted phosphoprotein 1, osteopontin	NM_009263	1.91	0.23
27	Spondin 2	BB007686	1.21	0.08
28	Tenascin C	NM_011607	1.53	0.04

Mouse mesangial cells(MES-13) were treated with low glucose(Control; LG, 5 mM), and TGF-β1(LG+10 ng/ml TGF-β1) for 24h, and transcriptome analysis was performed with affymetrix U430 2.0 genechips as described in methods. The genes whose expressions are regulated at least 1.5-fold+/-set up or down versus control are presented.

latory element-luciferase reporter activity for IFN-γ signaling by transient transfection in MES-13 cells after treatment with TGF-β1 for 24h. The luciferase-reporter activity in the pSTAT3-TA-LUC, pGAS-TA-LUC, and pISRE-TA-LUC transfected MES-13 cells are significantly increased ~5-8 folds(*, $p<0.05$: **, $p<0.01$; n=4) by TGF-β1 against untreated cells(Figure 3).

TGF-β1 increases ROS generation and apoptosis of mesangial cells

Exposure of mouse mesangial cells to high glucose and glucosamine for 48h increases ROS generation and the expression of TXNIP and apoptosis[5]. TGF-β1 also increases caspase 3 activities and causes cell death in renal mesangial cells[19]. Therefore, we examined whether TGF-β1 induces ROS production in MES-13 cells. Treatment of MES-13 cells with TGF-β1 for 24 h increases ROS production significantly($p<0.05$), which corresponds to TUNEL positive cells(Figure 4A and 4B). However, the expression of TXNIP mRNA is reduced by TGF-β1 after 24 hours(Figure 1). In our previous studies, we observed that TXNIP is an early response gene to high glucose and glucosamine and that stable overexpression of TXNIP in mouse mesangial cells sensitizes the cells to oxidative stress, ECM expression and apoptosis[5]. Therefore, we investigated the time-course of TGF-β1-mediated TXNIP and TRX expression in MES-13 cells. As shown in Figure 4C,

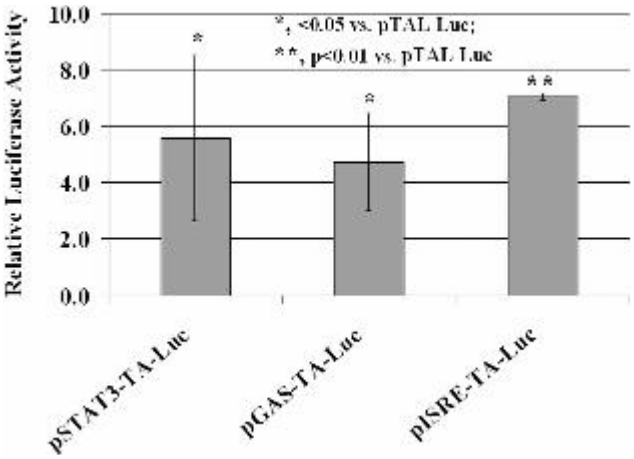

Figure 3: TGF-β1 induces transcriptional activation of interferon-γ signaling pathway

Plasmids of pSTAT3-TA-Luc, pGAS-TA-Luc, and pISRE-TA-Luc were transfected into mouse MES-13 cells as described in Methods using Lipofectamine™ 2000 Kit (Invitrogen). The luciferase activity was measured after treatments of the cells with or without TGF-β1 for 24 h as described in Methods. Significant increases in the luciferase activity are observed for all three reporter plasmids, pSTAT3-TA-luc, pGAS-TA-Luc, and pISRE-TA-Luc versus respective controls without TGF-β1, (n=4; *p<0.05; **P<0.01 versus control)

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TGF-β1 increases TXNIP level at 4h and then decreases at 24 and 48h. TRX expression is not altered. On the other hand, IGF-1, which is a hypertrophic and survival factor for mesangial cells, decreases TXNIP expression and increases TRX level at 48 h(Figure 4D).

Genetic cross-talks among TGF-β1, HG and GlcN induced gene expression

TGF-β1 plays a central role in the development and progression of diabetic nephropathy; however, chronic hyperglycemia is recognized as a culprit for the development of diabetic vascular complications. Therefore, we analyzed the potential genetic cross-talks among TGF-β1, HG and the HBP flux in mouse MES-13 cells. Comparing the TGF-β1-mediated transcriptome patterns to HG- and GlcN-induced transcriptome profiles[5], we found that several TGF-1, HG and GlcN-regulated genes in MES-13 cells overlap. A list of the 13 genes commonly targeted by TGF-β1, HG and GlcN is shown in TABLE 4. Among the 13 genes targeted by HG, GlcN and TGF-β1 commonly, ELK3, follistatin, interferon activated gene 205, RhoB, procollagen type 1 α1, secreted phosphoprotein 1(osteopontin), and PAI-1 are up-regulated by all three agents while the GC/GT binding transcription inhibitor krupple-like factor 15 is down-regulated. TXNIP, glutathione S-transferase theta 1, inhibitor of DNA binding 2 and 3 genes are positively regulated by HG and GlcN, but negatively regulated by TGF-β1. These genes are involved in ECM synthesis, interferon γ and follistatin/activin signaling[29] and mediates DNA-protein interaction and ROS generation in various tissues including the mesangial cell. Therefore, they may represent an

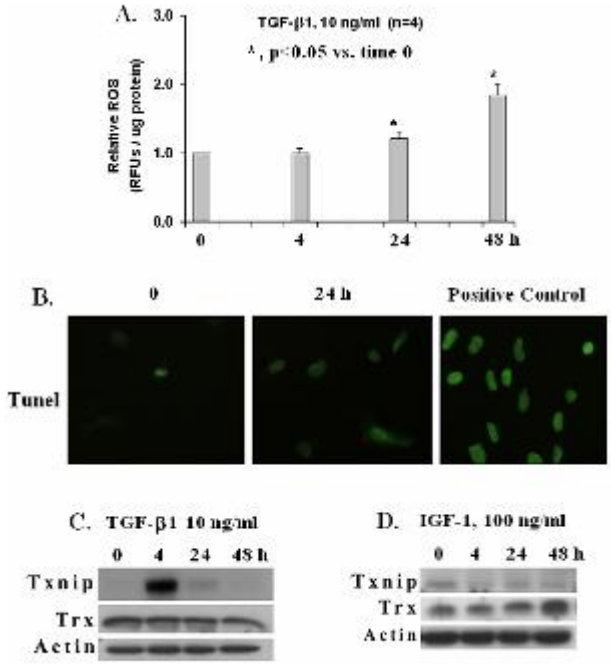

Figure 4: TGF-β1 induces reactive oxygen species (ROS) generation, apoptosis and TXNIP expression in MES-13 cells

(A) ROS measurement. Fluorescence-based thiol-reactive green CM-H_2DCFDA assay for the detection of ROS production was performed with 10 ng/ml TGF-β1 for different time periods (0-48h) in MES-13 cells as described in Methods. The symbol(*) represents p<0.05, n=4. (B) Tunnel assay: TGF-β1 induced apoptotic cells were detected by TUNEL positive cells after 24 h as compared to controls at time 0 as described in Methods. A representative of three different experiments is shown. (C-D) Western blots: MES-13 cells were treated with 10 ng/ml TGF β1 or 100 ng/ml IGF-1 for different time periods (0-48 h) and TXNIP, TRX and actin levels were detected by ECL on Western blots. Representative blots of n=3 are shown here.

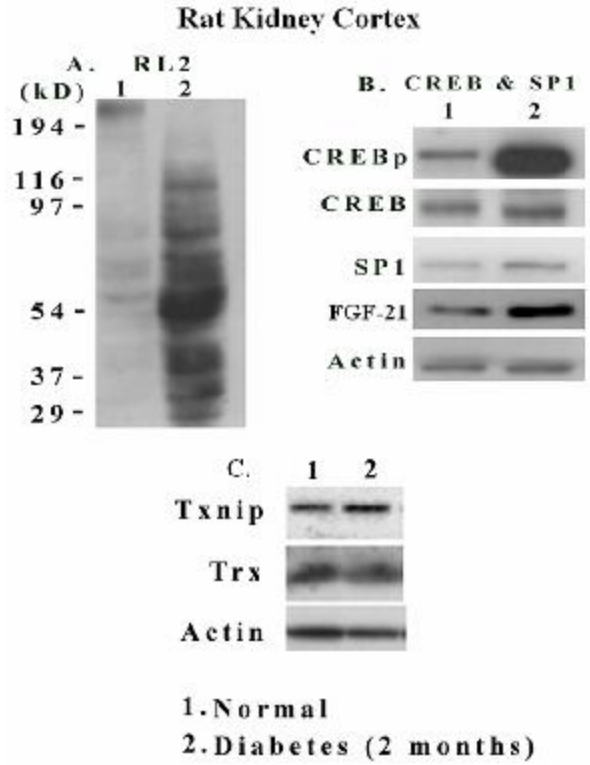

Figure 5. Diabetes increases hexosamine flux and TXNIP expression in the rat kidney

Protein extracts (30 μg) in RIPA buffer from renal cortex of normal and 2 months diabetic rats were subjected to 12.5% polyacrylamide SDS-PAGE and Western blotting. (A) Protein Ser/Thr-O-GlcNAcyaltion level (as a measure of increased HBP flux) was detected by RL2 antibodies; (B) Western blots of CREB(S133) phosphorylation, total CREB, Sp1 and FGF-21; (C) TXNIP and TRX expression in the kidney. Equal protein loading was visualized by actin. Representative blots of n=5-6 are shown here

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important networking channel to communicate the genetic cross-talks between high ambient glucose, HBP flux and TGF-β1 to induce oxidative stress, apoptosis and aberrant gene expression in renal mesangial cells under diabetic conditions.

In support of this hypothesis, we observed that, in streptozotcin-induced 2 months diabetic rats, the HBP flux is increased in the renal cortex as revealed by RL2 antibodies, which correlates with an increased TXNIP expression and up-regulation of transcription factor CREB phosphorylation and Sp1 expression(Figure 5A-C). Furthermore, TXNIP expression is also increased, not only in the kidney, but also in the diabetic retina(data not shown), which is a target of oxidative stress and ECM accumulation in diabetes. TRX is down-regulated while actin expression is not changed. The expression of FGF-21, which is one of the highest induced genes in mesangial cells by TGF-β1(Figure 1), is also increased in the kidney diabetic rats.

DISCUSSION

TGF-β1 has been identified as a common mediator of the principal lesions of diabetic nephropathy such as renal hypertrophy and accumulation of extracellular matrix proteins[37,40,47] in the glomerular mesangium. However, its effects on global gene expression and regulatory mechanisms in renal mesangial cells are not fully understood. To our knowledge, the present study represents the first TGF-β1-induced transcriptome profile of glomerular mesangial cells. We employed an in vitro model of mouse mesangial cells to investigate the genetic effects of TGF-β1 and its interaction with HG and GlcN on global patterns of mRNA expression, ECM regulation and signal networks. A total number of 264 genes were found to be regulated by TGF-1 treatment, which covered across 44 physiological processes, 31 metabolic functions, and 11 major signaling pathways in mouse MES cells. Both activation and repression of gene expres-

TABLE 4: Common gene regulated by HG, GlcN and TGF-β1 in mesangial cells

Sl #	Gene name	Accession #	HG (fold change)	se	GlcN (fold change)	se	TGF–β1 (fold change)	se
1	ELK3, member of ETS oncogene family	BC005686	2.06	0.03	1.68	0.23	1.83	0.23
2	Follistatin	BB444134	4.2	0.43	1.94	0.23	2.02	0.23
3	Glutathione S-transferase, theta 1	BC012254	1.92	0.13	2.48	0.23	-1.97	0.23
4	Inhibitor of DNA binding 2	NM_010496	1.86	0.14	3.17	0.23	-1.79	0.23
5	Inhibitor of DNA binding 3	NM_008321	2.85	0.2	2.66	0.23	-2.15	0.23
6	Interferon activated gene 205	AI481797	2.15	0.06	2.54	0.23	2.6	0.69
7	Kruppel-like factor 15	BC013486	-3.51	0.23	-2.39	0.23	-2.09	0.23
8	Procollagen type 1, α1	BI794771	3.67	1.33	3.14	1.36	1.55	0.13
9	Ras homolog gene family, member B, RhoB	BC018275	3.65	0.16	2.08	0.23	2.04	0.23
10	Secreted phosphoprotein 1, osteopontin	NM_009263	2.96	0.3	1.76	0.23	1.91	0.23
11	Serine (or cysteine) proteinase inhibitor, clade E, member 1 (PAI-1)	NM_008871	9.57	1.7	1.75	0.23	5.22	0.23
12	Serum/glucocorticoid regulated kinase	NM_011361	2.3	0.06	-2.72	0.23	2.25	0.23
13	Thioredoxin interacting protein	AF173681	18.83	2.89	9.91	0.23	-3.4	0.23

sion by TGF-β1 use the same set of activated Smad proteins, such as phosphorylation of smads 2/3 and binding to Smad 4 to translocate the proteins from cytosol to the nucleus while smad 7 is an inhibitory protein of TGF-β1 mediated gene transcription (Miyazawa et al. 2000). In this study a direct regulation of smad gene expression is not observed but the activation of ERK pathway, which cross-talks with Smad pathway is observed(Massague & Shi 2000). TGF-β1 increases the level of other growth factors, such as connective tissue growth factor, platelet derived growth factor B and fibroblast growth factor 21, and this will further compound the overall intracellular signaling mechanism(s) and cell growth response to TGF-1 signaling pathways in chronic diseases of diabetes mellitus. Both CTGF and PDGF are known to induce mesangial cell growth, hypertrophy and ECM gene expression in mesangial cells. FGF-21 has been shown to express in the liver and overexpression of FGF-21 in transgenic mice increases glucose uptake in adipocytes and protects animals from diet-induced obesity and lowers blood glucose and triglyceride levels[21] and pancreatic β-cell apoptosis[50]. The role of FGF-21 in the kidney is not understood at present although its expression is highly induced by TGF-β1 in mesangial cells and in the diabetic kidney. The increased expression of $p21^{Cip1}$ by HG, GlcN and TGF-β1 in mesangial cells and diabetic kidney will further lead to cell cycle arrest at G1/S phase leading to cell growth and hypertrophy[11,20].

TGF-β1 regulates interferon γ signaling pathways, which include STAT3, GAS and ISRE(Figure 3). HG and GlcN have also been shown to regulate a number of interferon-inducible genes and GAS and ISRE signaling pathways but not STAT3[5]. Interferon-γ exerts a pleiotropic effect in mesangial cell under inflammatory glomerular disease, which is mediated by STAT 1 and 2 with increased MAPK and GAS activity[6,33,38]. PDGF induced mesangial cell replication and collagen IV expression has been shown to correlate with an increased expression of pSTAT3 and pSmad1 on cultured mesangial cells[46].

As described above, TGF-β1 is a fibrotic cytokine that induces tissue scarring in diabetic kidneys due to an increased production and reduced degradation of ECMs leading to their excessive accumulation in the mesangium and basement membrane. TGF-β1 increases the expression of a number of extracellular matrix related genes in mesangial cells and these include fibronectin, laminin, collagen subunits and integrins(TABLE 3). Along with the increase in the expression of the genes, post-translation modification of the proteins by glycosylation also retards their rate of degradations[19]. TGF-β1 increases the expression of I-branching antigen gene GlcN-T2, which transfers terminal N-acetylglucosamine to glycoproteins and glycolipids, and correlates with an increase in WGA binding to mesangial cells and laminin β1. Tunicamycin, an inhibitor of N-linked protein glycosylation, blocks laminin β1 binding to WGA(Figure 2). Furthermore, PAI-1 mRNA is increased by TGF-β1 and also by high glucose and glucosamine(TABLE 4) suggesting that ECM accumulation is not only due to increased synthesis but also contributed by reduced degradation. TGF-β1 also increases the expression of other ECM species, which are otherwise not known to present in mesangial cells under normal conditions, such as laminin β3 and laminin γ2. Laminins are heterotrimers of αβγ subunits comprising of ~16 laminin types with 5α, 3β and 3γ isoforms[2]. In mesangial cells, laminin β1or β2 and γ1 are known to express with a modified form of the α4 polypeptide to form laminin 8(laminin 411) and laminin 9(laminin 421)[10,14]. Laminin β3 and γ2 with α3 forms the laminin 5(laminin332) isoform, which is present in the endothelial basement membrane suggesting TGF-β1 induction of aberrant laminin isoforms. Osteopontin expression is increased by high glucose, glucosamine and TGF-1 in mesangial cells(TABLE 4), and its up-regulation in diabetic kidneys has been reported by other investigators in microarrays[18,45].

A unifying mechanism for the development of vascular complications of diabetes has been proposed, which involves various glucose metabolic pathways, such as (i) mitochondrial oxidative stress, (ii) polyol pathway, (iii) advanced glycation end-product formation, (iv), protein kinase C pathway and (v) HBP flux[3,4]. A consequence of derailed glucose metabolism is the generation of ROS and modified products of proteins, DNA, and lipids in diabetes[30]. Both increased production of oxidants and a decreased

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action of antioxidants play a role in inducing oxidative stress in the glomeruli of diabetic kidneys. α-lipoic acid, an antioxidant, is effective in reducing high glucose and glucosamine mediated ROS generation and ECM expression in mesangial cells and prevents them from undergoing apoptotic demise[44]. α-lipoic acid also normalizes laminin γ1 and fibronectin levels in the diabetic rat kidney supporting a role for oxidative stress in ECM accumulation in diabetic glomerulopathy. Several effects of high glucose and glucosamine on ECM expression and mesangial cell death are further mediated by TGF-β1[19,43], and here we show that TGF-β1 itself increases ROS generation and apoptosis of mesangial cells(Figure 4). One mechanism by which high glucose and glucosamine induce ECM expression and mesangial cell death is via an induction of TXNIP expression and increased oxidative stress(Cheng et al. 2006). However, TGF-β1 decreases TXNIP mRNA levels by ~3.5-folds after 24h incubation (Figure 1). Initially, we interpreted the data as a cellular defensive mechanism since many of the genes commonly induced by high glucose and glucosamine such as glutathione S-transferase theta 1, inhibitors of DNA binding 2 and 3, are decreased by TGF-β1. However, in our previous studies, we demonstrated that TGF-β1 is much more potent than high glucose and glucosamine to induce ECM expression and that TXNIP is early response gene to high glucose and glucosamine treatment[19,43]. TGF-β1 indeed increases TXNIP expression at 4 h and then decreases at 24 and 48 h(Figure 4C) while IGF-1, which is a survival factor for mesangial cells[20], reduces TXNIP expression suggesting that TXNIP may cause cell death[32]. Thus, TXNIP represents an early response gene to high glucose, glucosamine and TGF-β1 and could be considered as a biomarker or inducer of oxidative stress, ECM expression and apoptosis of renal cells in the diabetic kidney.

The cellular thioredoxin system includes TRX, TRX reductases, NADPH, and thioredoxin peroxidases, which play an important role in balancing the redox potential of protein cysteine groups and direct scavengers for intracellular reactive oxygen species, such as H_2O_2[8]. TGF-β1 does not have a direct effect of TRX expression; however, as indicated above, the expression of its endogenous inhibitor, TXNIP, is increased(Figure 4C). Therefore, the activity of TRX may be regulated by an increased expression of TXNIP rather than TRX expression itself under excess glucose, HBP flux and TGF-β1 as seen in diabetes. TXNIP also plays an important role in cardiovascular disorders(Schulze et al. 2004), NK cell survival[25], ECM gene expression[5,22], diabetic neurons[36] and apoptosis of insulin secreting β-cells via an increased oxidative stress pathway[32]. At present, we do not know the molecular mechanism(s) of high glucose, glucosamine or TGF-β1 increases TXNIP expression[31]. TXNIP promoter contains a carbohydrate response element(ChoRE) and that glucose-induced human TXNIP promoter reporter gene expression in pancreatic β-cells requires this sequence motif[31]. Among the known ChoRE-binding proteins, upstream stimulatory factors(USF 1 and 2) are targets of the hexosamine pathway and USF-1 has been shown to mediate hexosamine-induced TGF-β1 gene expression via ChoRE[49,52]. Within the ChoRE of the promoter of TXNIP contains two e-box sequences with CpG islands and they have been shown to be targets of DNA methylation and gene silencing by epigenetic mechanisms[1]. Whether CpG demethylation and corresponding histone acetylation/methylation participate in the regulation of TXNIP gene expression in mesangial cells in response of high glucose, glucosamine and TGF-β1 as well as in the diabetic kidney remains to be investigated.

In conclusion, an analysis of the transcriptomic profile of TGF-β1-mediated gene expression in mesangial cells demonstrates that several genes known to be involved in diabetic glomerulopathy are differentially regulated by TGF-β1. The expression of ECM genes, I-type antigen enzyme GlcN-T2, protein glycosylation and ROS generation are increased by TGF-β1. Furthermore, TGF-β1 induces the expression of other fibrotic growth factors such as CTGF, PDGF-B and FGF-21 in renal mesangial cells. A comparison of the mRNA expression profile of TGF-β1 in renal mesangial cells with that of high glucose and HBP flux reveals that TXNIP, PAI-1 and matrix genes are commonly targeted by these agents and cause oxidative stress, ECM accumulation and apoptotic demise of mesangial cells. TXNIP may be considered as an early response gene/biomarker or a

mediator of oxidative stress in diabetic glomerulopathy.

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