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DECORIN GENE TRANSFER INHIBITED THE EXPRESSION OF TGFβ1 AND ECM IN RAT MESANGIAL CELLS

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Abstract

Objective: To explore the regulative role of decorin on the ECM gene-expression in diabetic nephropathy, recombinant adenovirus expressing rat decorin (Ad-decorin) was constructed to further investigat the effects of decorin overproduction on the expression of TGFβ1 and ECM in rat mesangial cells (RMCs) in high glucose condition.

Methods: The recombinant decorin adenovirus and lacz adenovirus(Ad-lacz), as a control, were constructed. RT-PCR, restriction enzyme digestion, western blot and gene sequence were used for validating correctness of Ad-decorin. MTT was used to examine the biological function of decorin (decorin expressed by Ad-decorin transduced CHO cells was used to interact with TGFβ1 which can inhibit the proliferation of Mv1Lu cells). Then Ad-decorin was transferred into rat mesangial cells cultured in high-glucose (450mg/dL) media and Ad-lacz was as the control transducer. TGFβ1, decorin, collagen IV, fibronectin, lamnin and tenascin mRNA in RMCs at 24, 48 and 72 hours after Ad-decorin infection were determined with RT-PCR. The distribution and expression of TGFβ1 protein was detected in RMCs at 96 hours after Ad-decorin infection by immunoperoxidase cell staining.

Results: RT-PCR, restriction enzyme digestion, western blot and gene sequence all confirmed that Ad-decorin could express correct decorin mRNA and protein. MTT showed that decorin protein expressed by Ad-decorin-transfected CHO cells abrogated the inhibitive effect of TGFβ1 on the proliferation of Mv1Lu cells. Decorin mRNA significantly increased in Ad-decorin transduced RMCs at all the observed time points, reached the peak at 24 hours(2.2-fold, $P<0.05$) and the overexpression lasted to the end of the observation at 72hours(1.7-fold, $P<0.05$) compared to that in Ad-lacz transduced RMCs. Meanwhile, TGFβ1 mRNA level began to fall at 48 hours (-20%, $P<0.05$) in Ad-decorin transduced RMCs and went to the valley at 72 hours (-46, $P<0.05$). ECM components, such as teascin, laminin, fibronectin and collagen IV, were reduced notably in the Ad-decorin transduced RMCs from the 48 hours to the end of study versus those in the Ad-lacz transduced RMCs. Cellular immunohistochemistry further comfirmed that the Ad-decorin transduced RMCs produced much less TGFβ1 compared with the Ad-lacz transduced RMCs.

Conclusion: The constructed recombinant decorin adenovirus can highly efficiantly express biologically active decorin. Overexpression of decorin down-regulates the expression of TGFβ1 and ECM components from RMCs. These results suggest that overexpression of decorin may be one of the theraputic approaches to diabetic nephropathy.

INTRODUCTION

Diabetic nephropathy (DN) is associated with the accumulation of extracellular matrix (ECM) proteins in the glomerulus and is represented morphologically by thickening and expansion of the glomerular basement membrane and the mesangium. Hyperglycemia is the primary etiologic factor in the metabolic abnormalities and vascular complications of diabetes. Prolonged exposure to high glucose is an important contributor to the development of diabetic nephropathy both in types 1 and 2 diabetes [1, 2, 3]. Although the mechanisms underlying the effects of chronic hyperglycemia on the kidney are not fully understood, TGFβ1 is now considered to be a key molecule that aggravates diabetic nephropathy [4, 5, 6].

Several lines of evidence revealed critical roles of TGFβ1 during the progression of glomerular lesions in diabetic nephropathy: 1) TGFβ1 expression is upregulated by glucose and enhances extracellular matrix (ECM) accumulation in mesangial cells [7] ; 2) TGFβ1 expression levels are markedly increased in mesangial areas in animals or in patients after the onset of diabetic nephropathy [8]; and 3) importantly, neutralization of TGFβ1 actions with a specific antibody suppresses glomerular hypertrophy as well as sclerosis in vivo [9, 10]. A distortion of the balance between ECM synthesis and turnover may result in an abnormal ECM accumulation in the mesangium in diabetic nephropathy. TGFβ stimulates the synthesis of key extracellular matrix molecules including type I collagen, type IV collagen, fibronectin, and laminin. And TGFβ also decreases matrix degradation by inhibiting proteases as well as activating protease inhibitors [9, 11, 12, 13]. Although the pathogenesis of glomerulosclerosis is uncertain, it is likely that all three major cells of the glomerulus participate in the fibrotic process. Among the resident cells of the glomerulus,

The first two authors contributed equally to this study.

mesangial cells are primarily responsible for excessive ECM deposition [10] and ECM accumulation often appears to begin in the mesangium [14]. There is increasing evidence indicating that mesangial autocrine activation of TGFβ mediates the effects of high glucose concentrations [15].

All above provide one strategy to inhibit the progression of diabetic nephropathy. One such approach is the use of the endogenous proteoglycan decorin, which is refered to as the naturally occurring inhibitor of TGFβ [16]. Decorin is a small leucine-rich Proteoglycan, and consists of a 40 kD core protein and one glycosaminoglycan chain. It is known for its ability to interact with several matrix molecules, including various types of collagen, fibronectin, thrombospondin and growth factors. Most of these interactions are mediated by the core protein [17]. More importantly, there appears to be a role for decorin in the regulation of TGFβ activity [16, 18].

In the present study, we constructed a recombinant adenovirus of decorin gene to further investigate the functional ability of overexpression of decorin to block the selected effects of TGFβ1 in rat mesangial cells.

Methods

Extraction of Total RNA

Total RNA in the kidney of a 8weeks SD rat (Animal study center of Zhejiang University) was abstracted in accordance with the manufacturer's introductions. The RNA was washed with 75% ethanol and briefly vaculum-dried. In the end, the RNA was dissolved in DEPC-H_2O. The integrity of the total RNA was analyzed by 1% agarose gel electrophoresis alongside RNA marker, and the purity of the total RNA was checked by the ration of OD260/280.

cDNA Synthesis and Amplification of Decorin Gene

First-strand cDNA was synthesized by reverse transcription(RT) of 2 μg total RNA using oligo dT_{18} and 200u superscript II reverse transcriptase(Invitrogen) at 42 °C for 70 min according to the protocal. PCR were carried out in a final volume of 50μl with 2μl of denatured cDNA and 2.5u Taq DNA polymerase High Fidelity platinum(Invitrogen), 1μM of both primers and PCR buffer containing 1.5mm $Mgcl_2$ and 200μm of each dNTP. The olignucleotide primers(Sangong, China) were used as follows: 5'-GCAGATCTATGAAG GCAACTCTCGTCTT-3' (upstream) and 5'-CGAAG CTTGCTTACTTGTAGTTCCCAAG-3'(downstream). The upstream and downstream primers were designed to introduce a BglII and a HindIII restriction site (underlined), respectively. The amplification was performed for 30 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 70 °C for 1 minute before a 5 minutes' extension was carried out at 72 °C. The amplified PCR product (size:1083bp) was resolved by electrophoresis on 1% (w/v) agarose gels, and purified decorin gene was digested with restriction enzymes of BglII and HindIII.

Construction of Adenovirus-Decorin (Ad-decorin) Plasmid

Decorin gene fragment digested by BglII and HindIII was inserted into the pShuttle-CMV vector(Stratagene Company, USA) digested with same enzymes. The insert fragment was confirmed by restriction digestion and sequence analysis. Purified shuttle vector plus gene of decorin in sufficient quantity was linearized by PmeI restriction enzyme. For the recombination, the BJ5183-AD-1 cells (Stratagene Company, USA) were transformed with the linearized shuttle vector (containing the gene of decorin). A recombination event that took place in the bacterial cells resulted in the production of recombinant AdEasy plasmid DNA including decorin gene (Ad-decorin plasmid). And the transformed cell suspension was inoculated on LB agar plates with kanamycin (50μg/ml). After 18h, clonies were picked on the plates and identified by PCR with vector-primers firstly. The olignucleotide primers (Sangong, China) were used as follows: 5'-GAAGTGAAATCTGAATAATTTTGT (upstream), 5'-GTGGGGGTCTTATGTAGTTTTG, (downstream). The size of amplified PCR product is 1181bp. The clonies containing exact decorin gene were confirmed with digestion of PacI, BglII and HindIII restricion enzyme and PCR. Recombinant Ad-decorin plasmid was digested with PacI enzyme and purified.

The Ad-decorin plasmid or Ad-lacz (β-galactosidase gene) plasmid was transferred with calcium phosphate into Ad293 cells (Human adenovirus transformed kidney cells, Stratagene company, USA.), respectively. After incubation at 37 °C for 10 days, the Ad293 cells, which appeared cytopathic effects, were harvested to freeze-thaw and vortex for 3 times to produce virus. The resulting adenovirus was amplified and purified and plaque tittered on Ad293 cells. Finally, the titer of adenovirus was adapt to 1×10^{10} pfu/ml with phosphate-buffer saline (PBS). Another adenovirus vector containing β-galactosidase gene (Ad-lacz) alone was constructed by the same way as a control.

Identifying the Expression of Decorin in CHO Cells

Confluent CHO cells (Chinese Hamster Overy cells, the American Type Cell Culture Collection), 1.0×10^6/60 mm dish, were treated with 1.0×10^8 pfu Ad-decorin or Ad-lacz, in 2ml/60 mm-dish of serum free media. After 24h of incubation, the supernatant was discarded and cells were washed 2 times with PBS. 5ml of standard medium per 60 mm-dish was added. Uninfected cells, cultured in the same conditions, served an additional negative control. After 96h, the supernatant was collected to be frozen to dry powder. 200μl PBS was added into the powder, which was commixed to be detected with western blot. A volume of the commixed solution subjected under non-reducing conditions to 10% SDS-PAGE and proteins were then transfered to polyvinyle difluofride membrane (Immobilon Millipore). Membrane was blocked for 2h in blocking Buffer (TBS containing 5% non-fat dry, 2% BSA, o.1% Tween-20), incubated with rabbit anti-murine decorin (1:100, LF113, a generous gift from

Dr. Larry Fisher) in blocking Buffer and overnight at 4 °C. Following washing in TBS/Tween (50mM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.5), membrane was incubated with a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, 1 : 2000) for 1h at room temproatuve, washed in TBS-Tween and developped by the ECL chemiluminescence detection system(Amersham). Decorin mRNA in CHO cells was detected with RT-PCR described above by following primers: 5'-CCGCATCTCAGACACCAACATA-3' (upstream), 5'-TTCCCAAGTTGAATGGTAGAGC-3' (downstream). The product size of PCR was 477bp.

MTT Analysis for Bioactivity of Ad-decorin

Confluent CHO cells was treated as above. Mv1Lu cells (mink lung epithelial cells, the American Type Cell Culture Collection) was distributed as 4×10^3 cells/well of 96 well-plate. Meantime, RMCs (rat mesangial cells, the Cell Institute of Wuhan, China), which can autocrine TGFβ1 into the culture [19], were distributed in the 60mm-dish as 1×10^6 cells. The mixture of supernatant of RMCs and supernatant of conditioned CHO cells (infected by Ad-decorin or Ad-lacz or uninfected) were aliquoted at a volume added into the culture media of Mv1Lu cells. After incubation at 37 °C for 24h, 48h, 72h and 96h, 20μl MTT (5mg/ml in PBS) was added into the wells. 4h later, the supernatant and cells were mixed sufficiently after 150μl DMSO was added into the wells, which were examined the proliferation of Mv1Lu cells by absorbance at 570nm with microplate reader. The proliferation was expressed as OD.

Detecting Decorin, TGFβ1 and ECM by RT-PCR and Immunoperoxidase Cell Staining

Confluent RMCs (for RT-PCR) in conditioned medium including 450mg/dl glu, 2×10^5 cells/60 mm-dish, were treated with 2×10^7 pfu Ad-decorin (*High glu+Ad-decorin*) or Ad-lacz (*High glu+Ad-lacz*) in 2ml/60 mm-dish of serum free media. After 2h of incubation, the supernatant was discarded and cells were washed 2 times with PBS. 3ml of standard medium per 60 mm-dish was added. Uninfected cells, cultured in the 100mg/dl (Normal glu) conditions, 450mg/dl (*High glu*), 450mg/dl+anti-TGFβ1 antibody (High glu+ TGFβ1 Ab) served as additional normal, negative and positive control, respectively (Table 1). The cells were collected at 24h, 48h and 72h to detect mRNA of decorin, TGFβ1 and ECM components including fibronetin, laminin, tenascin and collagen IV with RT-PCR. Primers used for RT-PCR are shown in Table 2. The mRNA levels of target genes were expressed as the ratio of target gene mRNA IDV to GAPDH mRNA IDV.

Cultured RMCs (for immunoperodoxidase cell staining) grew on sterile glass cover slips, and were

Table1. The divided RMCs groups.

Groups	*High glu*	*High glu+ Ad-decorin*	*High glu+ Ad-lacz*	*High glu+ TGFβ1 Ab*	*Normal glu*
Glucose(mg/dl)	450	450	450	450	100
Ad-decorin	–	+	–	–	–
Ad-lacz	–	–	+	–	–
TGFb1Ab (30μg/ml)	–	–	–	+	–

Table2. Primers used for RT-PCR in RMCs.

Name	**Gi**	**Size**	**Primer Sequences**	
GAPDH	37590766	305bp	Sense:	5'- TGGTGAAGGTCGGTGTGAAC-3'
			Antisense:	5'- GGTGGTGAAGACGCCAGTAG-3'
Decorin	34864778	477bp	Sense:	5'-CCGCATCTCAGACACCAACATA-3'
			Antisense:	5'-TTCCCAAGTTGAATGGTAGAGC-3'
TGFβ1	11024651	362bp	Sense:	5'-ACTACGCCAAAGAAGTCACCC-3'
			Antisense:	5'-AAGCCCTGTATTCCGTCTCC-3‘
Fibronectin	56163	450bp	Sense:	5'-AGTCCACCGCCACCATCAA-3'
			Antisense:	5'-CATCAGTGAATGCCAGTCCTTT-3'
Laminin	57250	461bp	Sense:	5'-ATGCTTCACCCAAACACCAGG-3'
			Antisense:	5'-TCACAGCGTCGTCCAACCA-3'
Tenascin	558866	188bp	Sense:	5'-TGAACGAACTGCCCACAT-3'
			Antisense:	5'-GAAACCGTCTGGAGTAGCAT-3'
Collagen IV	1816633	178bp	Sense:	5'-GAGCCAACGCTTCAGCAGG-3'
			Antisense:	5'-CCTCCAGCTCCGATCCAACC-3'

treated as described above. After 96h of incubation, the RMCs were fixed for 5 minutes in -10 °C methanol, air dry, and the cells were blocked in normal blocking serum at 37 °C for 1h. Then the slips were washed with PBS×3 times followed by incubation with rabbit anti-rat TGFβ1 (1 : 200, Santa Cruz, S-146, California), at dilution 1 : 200, at room temperatue for 1h. Following washing with PBS×3 times, cells were incubated with a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, 1 : 2000) for 1h and washed in PBS. Finally, immunoreactive proteins were stained using DAB and took photos magnified with 10×40 times.

Statistical Analysis

Differences between groups were analyzed using LSD of one-way ANOVA analysis with SPSS 11.0. $P<0.05$ was considered significent. Data were expressed as means ± SD.

Results

Agrose Electrophoresis Analysis of Ad-decorin

There appeared two bands about 7kb and 1083bp after pshuttle-CMV-decorin was digested with BglII and HindIII restriction enzymes and a band 1181bp after pshuttle-CMV-decorin was amplified by primers of adenovirus vector described in methods. After digested with PacI enzyme, the recombinant adenovirus vectors were cutted into two bands of 30kb and 4.5kb (Fig. 1).

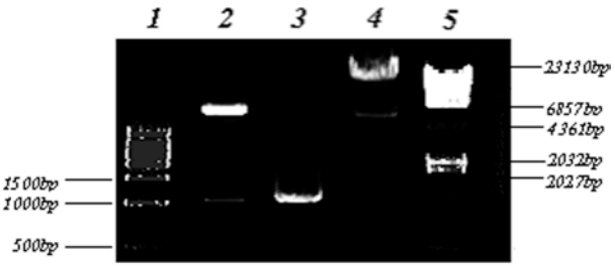

Fig.1. Agarose electrophorasis of primarily identifying of Ad-decorin plasmid. lane1: Marker; lane2: Two banes about 7kb and 1083bp were shown after digestion of pShuttle-CMV-decorin with BglII/HinsIII; lane3: A bane 1181bp was amplified by vector-primers from Ad-decorin plasmids; lane4: Two banes, 30kb and 4.5Kb were appearing when Ad-decorin plasmid was digested with PacI; lane5: Marker.

Decorin Expression Induced by Adenovirus Vector in Cultured Cells

RT-PCR was performed at 72h after cell infection to verify the expression of rat decorin mRNA in CHO cells. mRNA signal for rat decorin was detected to be expressed in Ad-decorin-infected CHO cells but was not found in Ad-lacz-infected cells or uninfected cells (Fig. 2A). The decorin protein in conditioned medium culture was assessed by western blotting, which demonstrated a band at 40kDa referring to decoirn core protein in Ad-decorin infected CHO cells as compared to Ad-lacz-infected or uninfected cells. (Fig. 2B).

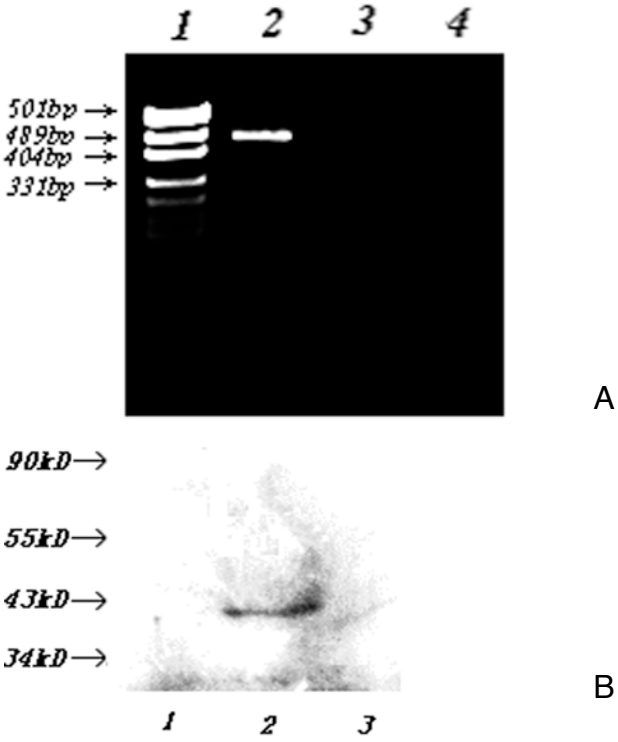

Fig.2. Identifying the expression of Ad-decorin in CHO cells. A: RT-PCR of decorin mRNA of CHO cells infected by Ad-decorin. There is a bane of 477 bp on lane of Ad-decorin in Agarose electrophorsis. (lane1)Marker. (lane2) Ad-decorin infected cells. (lane3) Ad-lacz infected cells. (lane4) uninfected cells; B: Western blot of decorin in supernatant using polyclonal against rat decorin. (lane1) Ad-Lacz infected CHO cells. (lane2) Ad-decorin infected cells and (lane3) unifected cells.

Decorin Abrogated the Inhibitive Effect of TGFβ1 on the Proliferation of Mv1Lu Cells

It is known that active TGFβ1 can inhibit the proliferation of Mv1Lu cells which can examined with MTT analysis. And the MTT analysis of our study showed that the proliferation of Mv1Lu cells was significiantly increased in cells treated with the mixture of the supernatant of RMCs and the supernatant of Ad-decorin infected CHO cells (Table 3, Fig. 3.).

Decorin Overproduction Inhibited TGFβ1 Expression in RMCs

RT-PCR revealed that high glucose concentration stimulated both decorin and TGFβ1 mRNA expression. And there were statistically significant increased expression of decorin and TGFβ1 mRNA in cells treated with high glucose compared with that in cells treated with normal glucose. Ad-decorin infected

Table3. Decorin abrogates the inhibitive effect of endogenous TGFβ1 of RMCs on the proliferation of Mv1Lu cells with MTT(unit: OD).

groups/time	24h	48h	72h	96h
Control	0.3612 ± 0.018	0.4678 ± 0.040	0.7904 ± 0.064♦◊	0.9332 ± 0.079♦◊
S-RMC	0.3302 ± 0.029	0.3376 ± 0.024	0.4520 ± 0.042*	0.5622 ± 0.044*
S-RMC + Ad-decorin	0.3546 ± 0.023	0.4476 ± 0.031	0.8014 ± 0.073♦◊	0.8624 ± 0.069♦◊
S-RMC + Ad-LacZ	0.2856 ± 0.019	0.4326 ± 0.028	0.3926 ± 0.022*	0.6013 ± 0.057*

Table3 : S-RMC : supernatants of rat mesangial cells.Compared with control (*P<0.05); Compared with the S-RMC group (♦P<0.05); Compared with the S-RMC+Ad-LacZ group(◊P<0.05). Values are mean ± SD.

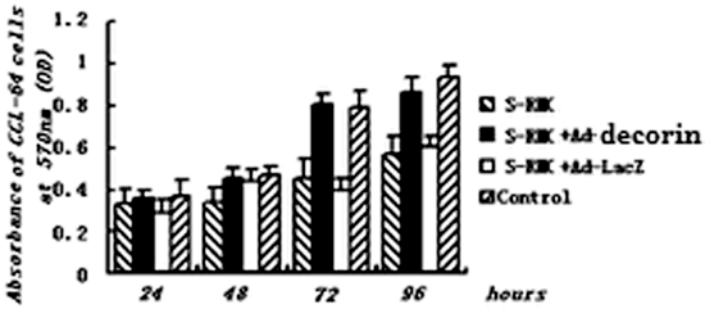

Fig.3. Absorbance at 570nm (unit is OD) of conditioned Mv1Lu cells treated with MTT and DMSO. Effects of decorin on inhibitive effect by endogenous TGFβ1 in supernatant of RMCs. Conditioned Mv1Lu cells were determined at day 24h, 48h 72h and 96h by MTT. Values are mean ± SD.

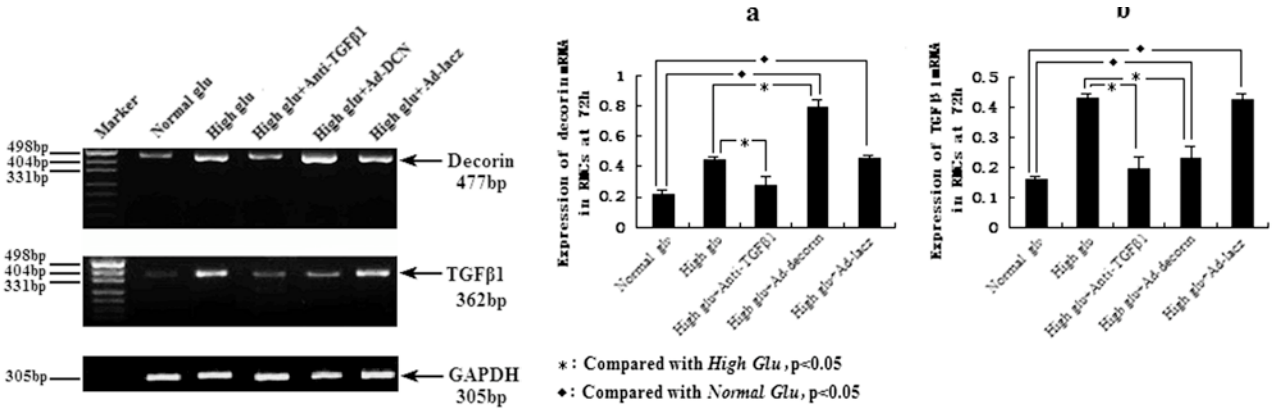

Fig. 4. Expression of Decorin mRNA and TGFβ1mRNA in RMCs by RT-PCR at 72h. (A) Agarose electrophorasis of decorin mRNA and TGFβ1mRNA in RMCs by RT-PCR at 72h. (B) a: The ratio of decorin mRNA IDV to GAPDH mRNA IDV by RT-PCR; b: The ratio of TGFβ1 mRNA IDV to GAPDH mRNA IDV by RT-PCR. Values are mean ± SD, n = 5.

RMCs had a significantly increasing of decorin mRNA expression as compared to uninfected cells in high glucose condition (2.2-fold at 24h, P<0.05; 2.1-fold at 48h, P<0.05; and 1.7-fold at 72h, P<0.05, respectively), and the decorin overproduction significantly down-regulated the expressing of TGFβ1 mRNA (-20% at 48h, P<0.05; and -46% at 72h, P<0.05, respectively). There were no differences of decorin and TGFβ1 mRNA expression between uninfected cells and Ad-lacz cells in the same condition (Table 4A and Fig. 4, 5).

Immunoperoxidase cell staining also showed that the steady-state level of TGFβ1 protein was significantly reduced in RMCs at 96h after Ad-decorin infection as compared to that in uninfected and Ad-lacz infected cells (Fig. 6).

Decorin Attenuated the Expression of ECM mRNA

The mRNAs encoding fibronectin, laminin, tenascin and collagen IV were determined by RT-PCR. Excitingly, a marked mRNA reduction for all ECM components above was observed at 48h in Ad-decorin cells as compared to Ad-lacz cells and uninfected high glucose conditioned cells and went to the valley at 72h (fibronectin –48%, P<0.05; laminin –44%, P<0.05; tenascin –50%, P<0.05; collagen IV –43%, P<0.05) (Table 5 B,C, Fig. 5). The inhibitive effect of Ad-decorin transfection on ECM expression was comparable to that of treatment with neutralizing anti- TGFβ1 an-

Table 4(A). The ratio of decorin mRNA IDV or TGFβ1 mRNA IDV to GAPDH mRNA IDV by RT-PCR.

Group	Observation point	24h	48h	72h	24h	48h	72h
		decorin			TGFβ1		
Normal glu		0.223 ± 0.031*	0.193 ± 0.032*	0.217 ± 0.024*	0.167 ± 0.006*	0.152 ± 0.010*	0.161 ± 0.007*
High glu		0.443 ± 0.023$^{\Delta}$	0.432 ± 0.016$^{\Delta}$	0.443 ± 0.023$^{\Delta}$	0.426 ± 0.006$^{\Delta}$	0.436 ± 0.11$^{\Delta}$	0.428 ± 0.016$^{\Delta}$
High glu+TGFβ1 Ab		0.436 ± 0.010$^{\Delta}$	0.332 ± 0.045*$^{\Delta}$	0.281 ± 0.053*	0.420 ± 0.005$^{\Delta}$	0.236 ± 0.049*$^{\Delta}$	0.193 ± 0.041*
High glu+Ad-decorin		0.990 ± 0.113*$^{\Delta}$	0.895 ± 0.059*$^{\Delta}$	0.790 ± 0.053*$^{\Delta}$	0.427 ± 0.011$^{\Delta}$	0.347 ± 0.039*$^{\Delta}$	0.231 ± 0.037*$^{\Delta}$
High glu+Ad-lacz		0.429 ± 0.021$^{\Delta}$	0.420 ± 0.004$^{\Delta}$	0.452 ± 0.024$^{\Delta}$	0.427 ± 0.026$^{\Delta}$	0.419 ± 0.133$^{\Delta}$	0.424 ± 0.022$^{\Delta}$

$^{\Delta}$: P<0.05, compared with Normal glu cells. *:P<0.05, compared with High glu cells.

Table.4(B). The ratio of fibronectin mRNA IDV or laminin mRNA IDV to GAPDH mRNA IDV by RT-PCR.

Group	Observation point	24h	48h	72h	24h	48h	72h
		fibronectin			laminin		
Normal glu		0.163 ± 0.034*	0.177 ± 0.016*	0.151 ± 0.016*	0.169 ± 0.015*	0.190 ± 0.014*	0.179 ± 0.012*
High glu		0.465 ± 0.026$^{\Delta}$	0.437 ± 0.008$^{\Delta}$	0.450 ± 0.019$^{\Delta}$	0.421 ± 0.006$^{\Delta}$	0.445 ± 0.028$^{\Delta}$	0.432 ± 0.034$^{\Delta}$
High glu+TGFβ1 Ab		0.419 ± 0.028$^{\Delta}$	0.241 ± 0.026*$^{\Delta}$	0.181 ± 0.024*	0.416 ± 0.006$^{\Delta}$	0.259 ± 0.006*$^{\Delta}$	0.220 ± 0.053*
High glu+Ad-decorin		0.416 ± 0.031$^{\Delta}$	0.363 ± 0.023*$^{\Delta}$	0.232 ± 0.006*$^{\Delta}$	0.415 ± 0.025$^{\Delta}$	0.382 ± 0.017*$^{\Delta}$	0.241 ± 0.023*$^{\Delta}$
High glu+Ad-lacz		0.426 ± 0.024$^{\Delta}$	0.418 ± 0.030$^{\Delta}$	0.443 ± 0.012$^{\Delta}$	0.426 ± 0.013$^{\Delta}$	0.419 ± 0.009$^{\Delta}$	0.431 ± 0.030$^{\Delta}$

$^{\Delta}$: P<0.05, compared with Normal glu cells. *:P<0.05, compared with High glu cells.

Table 4(C). The ratio of tenascin mRNA IDV or collagen IV mRNA IDV to GAPDH mRNA IDV by RT-PCR.

Group	Observation point	24h	48h	72h	24h	48h	72h
		tenascin			collagen IV		
Normal glu		0.162 ± 0.011*	0.183 ± 0.010*	0.170 ± 0.014*	0.111 ± 0.025*	0.126 ± 0.004*	0.138 ± 0.010*
High glu		0.432 ± 0.019$^{\Delta}$	0.414 ± 0.009$^{\Delta}$	0.428 ± 0.018$^{\Delta}$	0.349 ± 0.025$^{\Delta}$	0.385 ± 0.013$^{\Delta}$	0.351 ± 0.016$^{\Delta}$
High glu+TGFβ1 Ab		0.402 ± 0.009$^{\Delta}$	0.251 ± 0.018*$^{\Delta}$	0.171 ± 0.017*	0.330 ± 0.011$^{\Delta}$	0.180 ± 0.018*$^{\Delta}$	0.153 ± 0.006*
High glu+Ad-decorin		0.414 ± 0.028$^{\Delta}$	0.367 ± 0.031*$^{\Delta}$	0.0214 ± 0.002*$^{\Delta}$	0.342 ± 0.018$^{\Delta}$	0.316 ± 0.011*$^{\Delta}$	0.201 ± 0.017*$^{\Delta}$
High glu+Ad-lacz		0.421 ± 0.013$^{\Delta}$	0.415 ± 0.016$^{\Delta}$	0.406 ± 0.005$^{\Delta}$	0.346 ± 0.019$^{\Delta}$	0.327 ± 0.020$^{\Delta}$	0.337 ± 0.012$^{\Delta}$

$^{\Delta}$: P<0.05, compared with Normal glu cells. *:P<0.05, compared with High glu cells.

tibodies.

DISCUSSION

TGFβ is present in human glomeruli and has been associated with increased mesangial matrix in several glomerular diseases, including diabetic nephropathy [14]. And Our previous studies showed that expression of TGFβ1 in renal cortex of STZ-induced diabetic rat was markedly enhanced [20], and the level of TGFβ1 mRNA might be related with evolvation of diabetic nephropathy in human [21]. And in the present study we also found that in RMCs in high glucose condition, TGFβ1 mRNA production was notably enhanced from 24h and persisted to the end of the study versus that of cells cultured in normal glucose medium. These studies argue strongly in support of the hypothesis that overactivity of the TGFβ system in the kidney is a crucial mediator of diabetic renal hypertrophy and mesangial matrix expansion.

Several reports have indicated that TGFβ stimulates mesangial cell ECM production. In various studies including cultured mouse, rat and human mesangial cells or isolated perfused kidneys, TGFβ stimulates expression of types I, -III, and -IV collagen, laminin, fibronectin, and heparan sulfate proteoglycans [22, 23]. Our study also showed high glucose stimulated the expression of TGFβ1 as well as ECM components, such as type IV collagen, laminin, fibronectin and tenascin. This effect of high glucose could be abrogated by anti- TGFβ1antibody.

It has been assumed that there is a excess of TGFβ activity in models of glomerulonephritis, and that treatment with exogenous decorin will inhibit the excess activity of TGFβ and ameliorate the fibrotic state in these disorders [16, 24]. In contrast, in other forms of kidney diseases that are predominantly or exclusive-

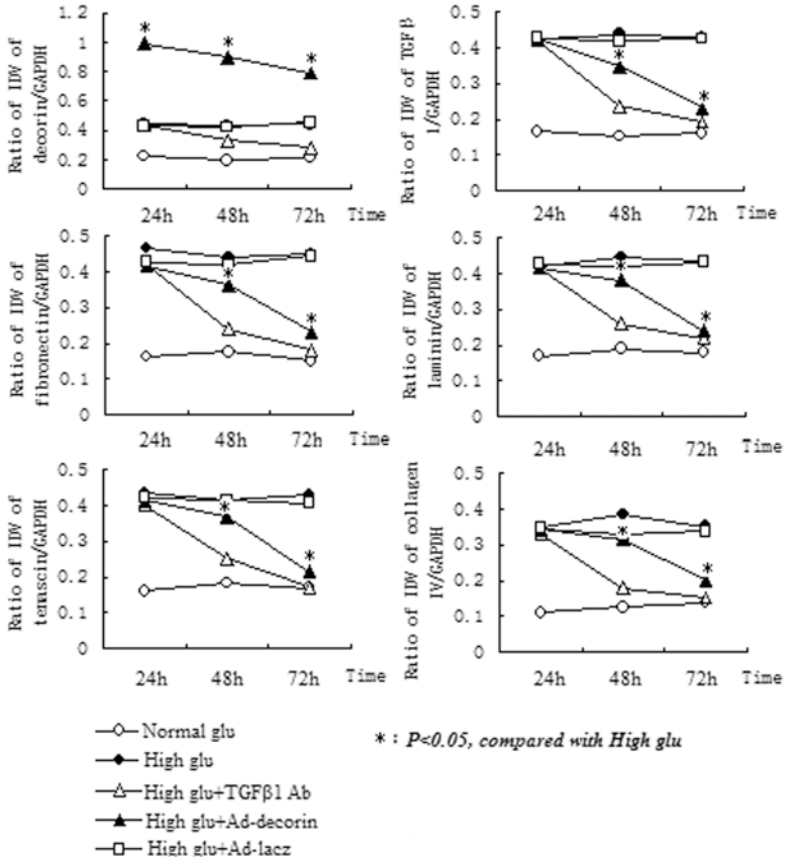

Fig. 5. Effect of decorin overexpression on the expression of TGFβ1 mRNA and ECM components of RMCs by RT-PCR at different time point. The mRNA levels of target genes were expressed as the ratio of target gene mRNA IDV to GAPDH mRNA IDV. Values are expressed as mean ± SD.

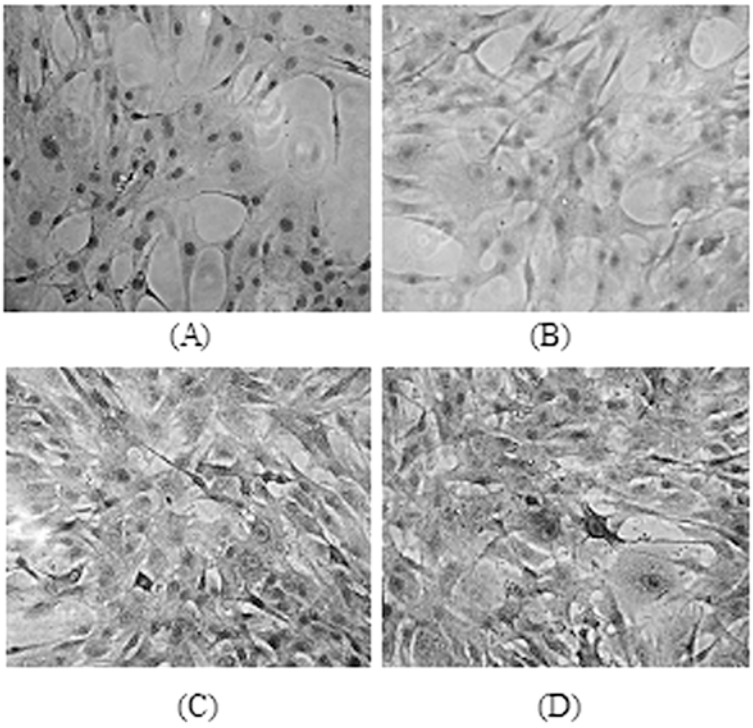

Fig. 6. Examination of the effect of decorin overexpression on the expression of TGFβ1 protein in RMCs after 96h in conditioned culture by cellular immunohistochemistry analysis. (A) Uninfected RMCs after cultured in 100mg/ml glucose medium. (B) Ad-decorin infected cells cultured in 450mg/ml glucose medium. (C) Ad-Lacz infected cells cultured in 450mg/ml glucose medium. (D) Uninfected RMCs cultured in 450mg/ml glucose medium.

ly non-inflammatory, a surfeit rather than a deficit in decorin expression pertains [25, 26]. Some findings also demonstrated increased decorin mRNA levels in glomerular mesangial cells under high ambient glucose conditions as well as in the kidnney cortex of streptozotocin-induced diabetes in the mouse [27], and the increased decorin may play a role in the progression of the human diabeitc nephropathy [28]. Whether exogenous decorin therapy will be beneficial in diabetic kidney disease remains to be established. In the present study, we constructed the Ad-decorin and to investigate whether decorin gene transfer has any benificial

role in ECM mRNA expression in RMCs cultured in high glucose. The recombinant decorin protein includes secret peptid of 16 amino acid, pre-peptid of 14 amino acid and the complete core protein. It was confirmed with PCR and western blot that CHO cells (naturaly not express decorin protein) infected with Ad-decorin could highly efficiently express 40kD decorin protein. Decorin is capable of bingding to TGFβ ligand with high affinity, resulting in the formation of inactive TGFβ-decorin complexes. Decorin therefore acts in an analogous manner as same to TGFβ neutralizing antibodies [29]. Since TGFβ1 can inhibit the proliferation of Mv1Lu cells, decorin protein in cultured Ad-decorin-infected CHO cells was used to interact with endogenous TGFβ1 produced by RMCs, and was proved that decorin in cultured CHO cells can abrogate the inhibitive effect of TGFβ1 autocrined by RMCs on proliferation of Mv1Lu cells as the result showed that the proliferation of Mv1Lu cells markedly increased. The result of MTT analysis indicated that the constructed Ad-decorin can express biologically active decorin.

Decorin mRNA significantly increased in Ad-decorin transduced RMCs at every observed point, and reached the peak at 24 hours and the overexpression lasted to the end of the observation (72h). Meanwhile, with TGFβ1 mRNA level falling at 48h after Ad-decorin transduction to RMCs, ECM components, such as teascin, laminin, fibronectin and collagen IV, were reduced notably in the Ad-decorin transduced RMCs from the 48 hours to the end of the study versus those in the Ad-lacz transduced RMCs and immunoperoxidase cell staining further comfirmed that the Ad-decorin transduced RMCs produced much less TGFβ1 compared with the Ad-lacz transduced RMCs. The possible mechanism for decorin down-regulating TGFβ mRNA level as follow: The TGFβ receptor signaling system can be triggered by overexpression of TGFβ, as evidenced by upregulation of the TGFβ type II receptor and activation of the downstream Smad signaling pathway [9]. With the falling of TGFβ1 protein level in Ad-decorin transduced RMCs, TGFβ type II receptor might be downregulation and the downstream Smad signaling pathway might be blocked, which lead to the decreasing of TGFβ mRNA level ultimately.

RT-PCR analysis indicated that high glucose upregulate decorin expression in RMCs, which may activate through the P1 promoter of decorin (and downregulate expression through the P2 promoter) [30]. It has found that decorin is present in low concentrations in the plasma of healthy individuals and decorin plasma levels rise in those patients who suffered from a progressive decline in their glomerular filtration rates in diabetic nephropathy. At earlier stages of the disease, increased plasma levels of decorin could not be recognized with the present methodology. However, decorin and possibly also the other small proteoglycans are efficiently cleared from the circulation. Intravenously injected decorin has been shown to be removed by the liver, possibly by the hyaluronan- and galactosaminoglycan-recognizing scavenger receptors of liver endothelial cells. Cultured macrovascular endothelial cells have also been shown to take up decorin by receptor-mediated endocytosis. These findings support the hypothesis that an increased secretion of decorin by diabetic glomeruli is compensated in part by an increased rate of endocytosis and that significant decorin levels in the urine are observed only when the filtered load overcomes the tubular capacity for reabsorption [31].

Furthermore, long-term antibody therapy in the db/db mouse, a spontaneous model of type 2 diabetes, virtually prevented the mesangial matrix expansion and preserved the creatinine clearance. However, the anti-TGFβ1 antibody did not reduce albuminuria [9]. And the limition of anti-TGFβ1 antibody (expensive, needing to manufacture and highly purify a recombinant protein, repeated parenteral administration) has been a serious obstacle to further development of this promising therapy [16]. Moreover, extreme reduction of TGFβ1 levels is pathological [32]. For example, mice who lack TGFβ1 protein, because of gene knockout, die soon after birth of an autoimmune-like illness. And loss of responsiveness to TGFβ1, due to mutation or shedding of TGFβ receptors, has been linked to malignant transformation in a number of human cell lines and tumors. Contrary, decorin can interact with TGFβ via its core protein, which is thought to fold into an arch-shaped structure [33]. And the binding of decorin to TGFβ is reversible, any TGFβ bound to decorin in tissue may form a reservoir of the growth factor [34]. In additional, adenovirus are capable of infecting a broad range of cell types and infection is not dependent on active host cells division. Additionally, high virus titers and high-level gene expression can be obtained, which are important considerations for protein production techniques in mammalian cells [12]. So we constructed the Ad-decorin, and the vitro results show that decorin gene suppress TGFβ1 gene expression, but not to zero and it is unlikely that gene therapy renders cells unresponsive to TGFβ1. Thus, suppressing TGFβ1 with decorin gene therapy would be safe. However, the long-term consequences of reducing TGFβ1 levels are unknown, and the possibility of adverse effects must be carefully studied.

Summary, the constructed Ad-decorin in our study can express active decorin protein in mammalian cells. And in RMCs treated with high glucose, the TGFβ1 mRNA and protein expression increaseing was bigpartialy suppressed by transfection of Ad-decorin. These results suggest that overproduction of decorin may be one of efficiently theraputic approaches to DN.

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