The Effects of Intra-Peritoneal Injection of Adenoviral Vectors Expressing Fibromodulin on Prevention of Diabetic Nephropathy

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**Abstract**--- Diabetic nephropathy (DN) is a major long-term complication of diabetes mellitus. Expression of TGF-β1 is elevated in the diabetic kidney. Fibromodulin a natural inhibitor of TGFβ1, may have healing properties. In this study, effects of intra-peritoneal injection of Adenoviral vectors expressing Fibromodulin on prevention of diabetic nephropathy were assessed. Fortyeight Sprague-Dawley rats were evenly divided into 4 groups: STZ-induced diabetic rats (diabetic-control), Fibromodulin adenovirus vector (Ad)-treated STZ rats (Ad-Fibromodulin), Ad-lacZ-treated STZ rats (Ad-lacZ), normal rats. At 10 weeks after STZ treatment, we measured urinary albumin excretion (UAE). We also measured kidney TGF-β 1 levels by Real-time PCR. Expression of Fibromodulin gene indicated by ReversTranscriptase PCR. There was a significant difference in UAE between four groups (P<0.05) and nonsignificant difference between normal group and Ad-Fibromodulin group. Fibromodulin expression confirmed by RT-PCR. TGF-β1 levels were significant differences in between 4 group (p<0.001). There were significant differences in Real-time PCR between four groups (p≤0.01).

**Keywords**--- Diabetic nephropathy, TGF-β1, Fibromodulin

I. INTRODUCTION

**DIABETIC** nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) [1]. Clinically, DN is characterized by progressive proteinuria, decline in kidney function accompanied by arterial hypertension, and increased risk of cardiovascular disease. DN is a prevalent cause of death in diabetes [2]. The pathogenic mechanisms of DN are not well understood, so there are few effective therapies [3]. Transforming Growth Factor-beta (TGF-β1) is a proinflammatory cytokine widely associated with the development of fibrosis in diabetic nephropathy[4]. TGF-β1 has multiple subtype, but TGF-β1 is most important in DN [5]. TGF-β1 is a potent mediator of fibrotic processes through stimulation of the synthesis of extracellular matrix components.

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TGF-β1 also induce albuminuria [6, 7] and also induce apoptosis in podocytes[8]. Elevated renal TGF-β1 mRNA and protein levels have been found in various animal models and the human form of diabetic nephropathy [9, 10].

This molecule has potential to be useful for therapeutic applications. Much effort has been made to find inhibitors of TGF-β1, such as TGF-β1 antibodies and soluble TGF-β1 type II receptor [11, 12]. Decorin is a natural inhibitor of TGF-β1 that found in the interstitial extracellular matrix [13]. Fibromodulin is a 59 kd collagen binding protein which is present in many types of connective tissues, cartilage, tendon, skin, sclera and cornea [15].FMOD is a small leucine-rich proteoglycan that along with other small leucine-rich proteoglycans, such as decorin, can interact with a number of different cell surface receptors, cytokines, ECM components, and growth factors, such as TGF-β ligands to modulate their activity side [16]. Fibromodulin, has not been studied in the setting of DN, so the aim of the paper is to investigate the effects of Fibromodulin gene in DN.

II. MATERIAL AND METHODS

A. Animlas

Adult male Sprague-Dawley weighing 200±30g were purchased and raised in our colony from an original stock of Razi institute (Tehran, Iran).The temperature was at 23±2 °C and animals kept under a schedule of 12h light: 12h darkness with free access to water and standard laboratory chow.

B. Protocol of Study

Seventy-two hours after the STZ injection, blood samples were taken from the tail vein and blood glucose was measured with Glucometer. Eight weeks after the STZ injection, 36 rats were randomly divided into 3 groups (n=12). One group (STZ-Fibromodulin group) received a 0.6 ml intraperitoneal injection of Ad-Fibromodulin. Another group (STZ-lacZ group) received a 0.6 ml (1 X 10⁹ PFU/ml) intraperitoneal injection of recombinant adenovirus encoding lacZ (1 X 10⁹ PFU/ml). The last group (STZ-PBS group) received a 0.6 ml in-traperitoneal injection of PBS. At this time, the 12 vehicle control rats (PBS-control) that were not induced with diabetes also received a 0.6 ml intraperitoneal injection of PBS. At 10 weeks after STZ injection, individual rats were placed in metabolic cages to obtain 24 h urine collections. The animals were then sacrificed, blood and kidneys were collected for analysis. The urine samples were kept at −20 °C until later measurements of creatinine (Jaffé assay) and albumin (competitive ELISA). Kidneys were quickly removed and thoroughly washed with saline to remove blood and kept at −80°C for reverse transcription polymerase chain reaction (RT-PCR) and Real-time PCR analysis. Kidney hypertrophy index (KHI) was calculated as

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the ratio of kidney weight (g) to body weight (kg). Urine albumin excretion (UAE) values were calculated as the ratio of urinary albumin/urinary creatinine (μg/mg).

C. RT-PCR for Fibromodulin

Total RNA was extracted from frozen renal tissue using RNaseqy Mini Kit (Qiagen Catalog no: 74104). The concentration of total RNA was quantified by nanodrop 2000 (Thermo scientific). In general, 1μg RNA,10 μl RNAse free water and 5X Qiagen OneStep RT-PCR Buffer were mixed with 1 μl NTPs (10 mM each; Qiagen), 1μl QIAGEN OneStep RT-PCR Enzyme Mix, 2μl forward primer, 2 μl reverse primer. The following PCR conditions for Fibromodulin were used: 52°C/30 min, 95°C C/15 min, followed by 40 cycles at 95°C C/45 s and 62°C/45 min, 72°C/1 min, 72°C/10 min and stopped at 4°C. The PCR condition for βactin, as an internal control, were used: 52°C/30 min, 95°C C/15 min, followed by 40 cycles at95°C C/45 s and 59°C/45 min, 72°C/1 min, 72°C/10 min. PCR product was separated with gel electrophoresis on a 1% (w/v) agarose gel and then visualized with ethidium bromide staining, under UV-light.

Fmod forward primer: 5’- TGA AGG CAG CAC CTG ACC GC-3’
Fmod revers primer: 5’- ACG CCT TGG CTT CTC CTG CC-3’ (amplification size:196 bp)

D. Real-time PCR

After extraction of total RNA from frozen tissue samples or cells, first-strand cDNA was generated for each sample with QuantiTect Reverse Transcription Kit (Qiagen Cat No: 205311). Quantitative real-time PCR analysis was carried out using an ABI 7300 sequence detector (Applied Biosystems). The PCR reactions were performed in 25μl volumes containing 12.5μl of QuantiFast SYBRGreen PCR Master Mix, 1μl of each forward and reverse primers, and cDNA corresponding to 100 ng RNA. PCR reactions for TGF-β1 and β-actin were performed using the following cycle conditions: 95°C/5 min, followed by 35 cycles at95°C C/10 s and 60°C/30 s.

TGF-β1 forward primer: 5’- GGT AAC CGG CTG CTG ACC-3’
TGF-β1 revers primer: 5’- GCC CTG TAT TCC GCC TCC TTG-3’ (amplification size:101bp)

E. Statistical Analysis

All the data were analyzed by SPSS version 19.0. The Mann-Whitney test was used to compare mean values between two groups. The Kruskal-Wallis or ANOVA test was used to compare mean values between more than two groups. The calculation of Spearman’s rank correlation coefficient was used to assess the relationship between quantitative parameters. Data are expressed as means SEM. All reported P values ≤0.05 was considered statistically significant.

III. Results

Fibromodulin expression confirmed by RT-PCR (FigI). TGF-β1 levels were significant differences in between healthy group and all three groups (p<0.001)(FigII). There was a significant difference in UAE between four groups (P<0.05) and nonsignificant difference between normal group and Ad-Fibromodulin group(FigIII).

Fig. 1 RT-PCR of Fibromodulin mRNA.
There is a band of 196 bp on lane of Ad-Fibromodulin in Agarose electrophoresis. (lane1) Marker,100bp. (lane2) Ad- Fibromodolin infected cells. (lane3) Non Template Control, (lane4) β actin band, 207bp.

Fig. 2 TGFβ1expression were elevated in the kidney of the diabetic-control group.* indicates significant difference compared to control group.

Fig. 3 Urin albumin to Urin creatinin ratio in control and treated rats. * indicates significant difference compared to control group.

IV. Discussion

TGFβ is present in human glomeruli and has been associated with increased mesangial matrix in several glomerular diseases, including diabetic nephropathy[17]. Other studies showed that expression of TGFβ1 in renal cortex of STZ-induced diabetic rat was markedly enhanced [18], and the level ofTGFβ1 mRNA might be related with evolvement of diabetic nephropathy.
in human [19]. Our data in the diabetic rat model indicate that Fibromodulin had a protective effect on DN. Observations in the Ad- Fibromodulin group suggest that Fibromodulin gene transfer via intraperitoneal injection markedly attenuates these pathological manifestations of diabetic. [20], the lower UAE level in the Ad- Fibromodulin group compared to diabetic-control indicate added amelioration of albuminuria with Fibromodulin therapy. This protective effect of Fibromodulin did not include any improvement in blood glucose levels, as the Ad- Fibromodulin group did not show any difference from diabetic controls. Furthermore, the therapeutic effect of Ad Fibromodulin was independent of the adenoviral vector, as the Ad-lacZ group did not show any improvements in albuminuria and KHI. The therapeutic mechanism of Fibromodulin in DN remains undefined. One possibility is the high affinity binding of Fibromodulin to TGF-β1 and the formation of TGF-β1 Fibromodulin complexes, which effectively inactivate TGF-β1 [21]. The other possibility is Fibromodulin can inhibit the expression of TGF-β1 mRNA, which has been confirmed in other studies. Our results are also consistent with these studies. We found that TGF-β1 mRNA was decreased in Ad –Fibromodulin group at the 10th week. However, this inhibition of TGF-β1 only appeared at the 10th week and it may be about to titer of Fibromodulin in the kidney. Perhaps only high titer of Fibromodulin can decrease the TGF-β1 expression. Real-Time PCR showed that TGF-β1 mRNA expression elevated in the kidney of the diabetic-control group TGF-β1 mRNA decreased at the 10th week after transfection in the Ad-Fibromodulin group compared to those in the diabetic-control group. Our results are also consistent with these results. In the present report, a recombinant adenovirus expressing Fibromodulin was tested for its therapeutic value in an animal model for diabetes. Ad- Fibromodulin shows potential as a therapeutic for human DN.

V. CONCLUSION

Intra-peritoneal injection of Adenoviral vectors expressing Fibromodulin may prevent diabetic nephropathy. Our suggestion is survey the molecular mechanism of nephropathy and SMAD signaling in the following study.

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REFERENCES
