Effects of Iridoid Total Glycoside from *Cornus officinalis* on Prevention of Glomerular Overexpression of Transforming Growth Factor Beta 1 and Matrixes in an Experimental Diabetes Model

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The present study was conducted to determine whether iridoid total glycoside from *Cornus officinalis* was effective in regulating expression of transforming growth factor beta 1 (TGF- β 1) and preventing overdeposition of extracellular matrix (ECM) in a diabetes state. An experimental rat model of diabetic nephropathy (DN) was successfully induced by one intraperitoneal injection of streptozotocin at a dose of 60 mg·kg⁻¹ and maintained for 12 weeks. All rats had free access to standard chow and water. Four groups: normal control, diabetic control, diabetic rats with aminoguanidine treatment and diabetic rats with iridoid total glycoside treatment were used in this experiment. All treatments were administered by intragastric gavage (ig). At the end of the experiment, serum was collected for ELISA determination of TGF- β 1 protein level; renal cortex was dissected for reverse transcription polymerase chain reaction (RT-PCR) analysis of its mRNA expression; and immunohistochemistry was introduced to observe ECM deposition. A significantly higher level of protein and mRNA expression of TGF- β 1, and also overdeposition of fibronectin and laminin was found in diabetic rats. Both iridoid total glycoside and aminoguanidine were effective in decreasing serum protein level and glomerular mRNA expression of TGF- β 1, and in preventing renal overdeposition of fibronectin and laminin. This study suggests that iridoid total glycoside is a beneficial agent for prevention and therapy of DN.

Key words *Cornus officinalis*; diabetic nephropathy; transforming growth factor beta 1; fibronectin; laminin

Diabetic nephropathy (DN), a common complication in patients with either type I or type II diabetes mellitus, has long been recognized to cause severe morbidity and mortality. It is also a major cause of chronic renal failure involved in dialysis therapy.^{1,2)} DN is structurally characterized with the early appearance of hypertrophy in glomerular and tubular components, the subsequent development of thickened glomerular and tubular basement membranes, and the progressive accumulation of extracellular matrix (ECM) components in the glomerular mesangium and tubulointerstitium.³⁾ The functional disorders were manifested by early microalbuminuria, renal hyperfiltration, hyperperfusion, and increasing capillary permeability to macromolecules, proteinuria and the end stage of renal failure (ESRF).⁴⁾

It has been postulated that DN occurs as a result of the interplay of metabolic and hemodynamic factors in the renal microcirculation. The main metabolic disorder of diabetes, chronic hyperglycemia, was accepted as a necessary prerequisite for the progression of DN. Hyperglycemia may act directly to damage the kidneys,⁵⁾ or may act indirectly through formation of advanced-glycation end products (AGEs),⁶⁾ activation of PKC pathway,⁷⁾ or causing abnormal expression of some important cytokines, among which TGF- β 1 was reported to be the most important in the onset and progression of DN. TGF- β 1 could stimulate mesangial cells uptaking glucose owing to overexpression of GLUT1, which leads to the acceleration of intracellular metabolic abnormalities in diabetes.⁸⁾ TGF- β 1 has been well proved to be a necessary prerequisite for the development of glomerular hypertrophy in streptozotocin-induced diabetic mice.⁹⁾ But the most important pathological effect of TGF- β 1 may be that it can advance the expression of many kinds of ECM¹⁰⁾ and reduce their degradation by inhibiting enzyme activity of the matrix

metalloproteinase (MMP) family.¹¹⁾ Both contribute to the accumulation of ECM in glomerular mesangium and tubulointerstitium, eventually causing glomerulosclerosis and tubulointerstitial fibrosis.

Iridoid total glycoside from *Cornus* was a complex consisting mainly of morroniside and loganin. It had been previously proved capable of inhibiting overformation of AGEs *in vitro* and *in vivo*. Furthermore, its effect on partially recovering levels of some vasoactive factors and cytokines such as NO, ET, TNF- α and sICAM in the diabetes state had been reported.^{12,13)} Through morphological study, we had also observed that glomerulosclerosis in an experimental diabetes model was apparently prevented and retarded. To further elucidate its possible effects and mechanisms, the present study was designed to determine whether glomerular expression of TGF- β 1, fibronectin, and laminin in diabetes rats induced by streptozotocin were restored to a certain degree by iridoid total glycoside treatment.

MATERIALS AND METHODS

Iridoid Total Glycoside Iridoid total glycoside was kindly provided by Jiangsu Zhongkang new drug fingerprint R & D Co., Ltd. Its purity was over 40% as determined by HPLC taking morroniside and loganin as its main compounds. It was freshly diluted to the desirable concentration with distilled water.

The Diabetic Model Male Sprague Dawley rats weighing 180–220 g from the animal center of Nanjing University of Traditional Chinese Medicine were used for this experiment. All rats were housed in an air-conditioned room at 23±2 °C with a 12 h cycle of light to dark. Food and water were allowed *ad libitum*. To establish a diabetes model, rats

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were given a single intraperitoneal injection of streptozotocin (STZ, 60 mg·kg⁻¹, Sigma Chemical Co, U.S.A.) which was freshly prepared with 0.1 mmol·l⁻¹ saline citrate buffer (pH 4.5). The level of fasting blood glucose was measured with a blood glucose analyzer three days later. Only rats with a blood glucose level of 16.7 mmol·l⁻¹ or higher were accepted as diabetic models according to the previous report.¹⁴⁾ Accepted rats were then randomly divided into three groups: diabetic control, diabetes rats treated with aminoguanidine (0.1 g·kg⁻¹), and diabetes rats treated with iridoid total glycoside (1.0 g·kg⁻¹), and at the same time a normal group of rats was introduced as control. All treatments were administered by intragastric gavage (ig). Successful development of DN was accomplished in 12 weeks.

Determination of TGF- β 1 in Serum by ELISA KIT Serum sample was collected at the end of the experiment and frozen at below -20 °C before analysis. All the analytic procedures were conducted according to the standard TGF- β 1 ELISA KIT (purchased from Oncogene Corporation) protocol. Briefly, a serum sample was first activated and then 100 μ l of the activated sample was tipped into a 96-well microplate and incubated at 37 °C for 60 min. After washing, 50 μ l of primary antibody was added followed by incubating at the same condition. Similarly, 100 μ l secondary HRP-conjugated antibody was later added and incubated for another 60 min. Finally, each well was allowed to react with colorimetric substrate, 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 50 μ l 1 mol·l⁻¹ of sulfuric acid and was read from a microplate reader at 492 nm. Absorbance of the samples obtained was subtracted from that of the blank well and the value was expressed as petagram per milliliter. The standard curve was constructed for quantitative analysis.

Immunohistochemistry Observation of Fibronectin and Laminin The left kidney was removed from each rat and cut into pieces with scissors which were then fixed in 4% formaldehyde. After a series of paraffin section procedures, immunohistochemistry detection of fibronectin and laminin was performed as follows. Briefly, after dewaxing, rehydrating and rinsing with PBS three times, the sections were digested with 0.2% trypsin for antigen retrieval. To block non-specific binding sites, 50 μ l normal goat serum was applied to each slide and incubated at room temperature for 10 min. Sections were then incubated with either goat anti-fibronectin or anti-laminin antibody (Fuzhou Maxim Biotech, Inc.) for 60 min at room temperature, washed in PBS, and incubated with biotinylated rabbit Ig for 30 min and then by avidin-biotin complex. Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride as a chromogen. The sections were at last visualized under a microscope for their color change and, if desired, counterstained and mounted for photograph and image analysis; six views were then randomly selected for calculating the average gray of the immunostaining (CMIAS 98A image analyzer, Beijing University of Aeronautics and Astronautics). A higher average gray indicated a lower intensity of immunostaining.

RT-PCR Determination of TGF- β 1 mRNA in Renal Cortex About 200 mg of renal cortex was dissected on ice and stored in liquid nitrogen before RNA extraction. Total RNA was extracted using Tripure isolation reagent (Roche Diagnostics Corp, Takara RNA PCR Kit (AMV) Ver.2.1) ac-

cording to the protocol. Purity of the acquired RNA was confirmed by electrophoresis and spectrophotometer from which RNA concentration was also read. Reverse transcription and polymerase chain reaction was performed using a RT-PCR Kit (Roche Diagnostics Corp, Takara RNA PCR Kit (AMV) Ver.2.1). RNA of different samples was adjusted to the same concentration of 0.25 μ g· μ l⁻¹. Four microliters of this RNA solution (1 μ g) was subjected for the first-stand cDNA synthesis the process of which was programmed on a Peltier thermal cycler (PTC-225, MJ Research, Inc, Watertown, Massachusetts, U.S.A.) at 30 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min, 5 °C for 5 min. Primers for TGF- β 1 and β -actin were commercially synthesized (ShenYou Biotechnology Lt Corp, Shanghai) according to the previous report as follows: TGF- β 1:¹⁵⁾ 5'-CGA GGT GAC CTG GGC ACC ATC CAT GAC-3' (sense), 5'-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3' (antisense); β -actin:¹⁶⁾ 5'-CGT AAA GAC CTC TAT GCC AA-3' (sense), 5'-AGC CAT GCC AAA TGT GTC AT-3' (antisense). The total volume of polymerase chain reaction was 30 μ l consisting of 4 μ l cDNA sample, 2 mM MgCl₂, 1U Taq polymerase, 0.33 μ M primer each, 0.25 mM dNTP mixture, and 1 $\times$ PCR buffer. Amplification for both genes was programmed at 94 °C for 5 min for the initial melt, followed by 32 cycles for TGF- β 1 and 28 cycles for β -actin of the following sequential steps: 94 °C for 40 s, 56 °C for 40 s, 72 °C for 80 s and 72 °C for 7 min. Ten microliters of PCR product was used for 1% (w/v) agarose gel electrophoresis with ethidium bromide staining followed by densitometric scanning on a microcomputer imaging device (GeneGenius, Syngene). The relative amounts of TGF- β 1 are expressed for normalization as ratios of the OD values for TGF- β 1 and β -actin PCR products.

Statistical Analysis All data were expressed as means $\pm$ S.D. Independent t-test on the SPSS package was adopted to analyze the statistical significance between groups. *p* values less than 0.05 were considered significant.

RESULTS

Effect on Serum TGF- β 1 Protein The quantity of each sample was determined from an established standard curve and expressed as pg/ml. Significant higher quantity of serum TGF- β 1 was determined in the diabetic control rats compared to that in normal rats, but was significantly decreased by either aminoguanidine or iridoid total glycoside treatment (Table 1).

Effect on the Deposition of Fibronectin and Laminin

Table 1. Effect of Iridoid Total Glycoside on Serum Protein Level of TGF- β 1

Group	<i>n</i>	Dose (g·kg ⁻¹)	TGF- β 1 (pg·ml ⁻¹)
Normal control	8	—	162.5 $\pm$ 61.6
Diabetic control	7	—	265.9 $\pm$ 164.2 [#]
Aminoguanidine	7	0.1	157.0 $\pm$ 30.5*
Iridoid total glycoside	8	1.0	180.6 $\pm$ 21.1*

Diabetes rats were induced by one intraperitoneal injection of STZ at a dose of 60 mg·kg⁻¹. All treatments were administered ig once a day for 12 consecutive weeks. Group aminoguanidine means the diabetic rats treated with aminoguanidine, and group iridoid total glycoside means the diabetic rats treated with iridoid total glycoside. Data are expressed by means $\pm$ S.D. [#]*p*<0.05, diabetic control versus normal control; **p*<0.05, treatments versus diabetic control.

Fig. 1. Effect of Iridoid Total Glycoside on Deposition of Fibronectin in the Kidney of Diabetes Rats

Deposition of fibronectin in the kidney was observed by immunohistochemical analysis and photographed under a light microscope. A, B, C and D represent normal control, diabetic control, diabetic rats with aminoguanidine treatment and diabetic rats treated with iridoid total glycoside, respectively. Fibronectin was positively stained light brown in glomeruli and tubulointerstitium of normal control. A much heavier staining indicating overdeposition was observed in the diabetic control animals, whereas it was prevented to a certain degree by either aminoguanidine or iridoid total glycoside treatment. Scale bar indicates 25 μ m.

Fig. 2. Effect of Iridoid Total Glycoside on Deposition of Laminin in the Kidney of Diabetes Rats

Deposition of laminin in the kidney was observed by immunohistochemical analysis and photographed under light microscope. A, B, C and D represent normal control, diabetic control, diabetic rats with aminoguanidine treatment and diabetic rats treated with iridoid total glycoside, respectively. Scale bar indicates 25 μ m.

Both fibronectin and laminin were positively stained in the glomeruli and tubulointerstitium of normal rats. After image analysis, a significantly stronger intensity of immunostaining (lower average gray) was observed in the diabetic control rats. Both aminoguanidine and iridoid total glycoside treatment attenuated the overdeposition of fibronectin and laminin in diabetic rats (Figs. 1—3).

Effect on Glomerular TGF- β 1 mRNA Expression RT-PCR analysis result showed a much stronger ethidium bromide staining in diabetic control rats than that in normal rats, while a weaker staining was noted in the diabetic rats with either aminoguanidine or iridoid total glycoside treatment (Fig. 4). Semi-quantitative analysis further confirmed a signifi-

Fig. 3. Calculated Average Gray for Fibronectin and Laminin Deposited in Kidney

Six views of each slide were randomly selected for calculation of the average gray. A higher average gray indicated a lower intensity of the immunostaining. FN and LN was the abbreviation of fibronectin and laminin, respectively. Data are expressed as means $\pm$ S.D. ^{##} $p < 0.01$, diabetic control versus normal control; ^{*} $p < 0.05$, ^{**} $p < 0.01$, treatments versus diabetic control.

Fig. 4. Electrophoresis Graph of PCR Products of TGF- β 1 and β -Actin

M stands for DNA marker, N1, 2 for normal control, C1, 2, 3 for diabetic control, A1, 2, 3 for diabetic rats treated with aminoguanidine and H1, 2, 3 for diabetic rats treated with iridoid total glycoside. The upper band at 409 bp represents TGF- β 1; the band below at 349 bp represents β -actin.

cantly higher expression level in diabetic control rats and a significantly inhibitory effect with both aminoguanidine and iridoid total glycoside treatment (Fig. 5).

DISCUSSION

The experimental diabetic model induced by one intraperitoneal injection of STZ with a dose of 60 mg $\cdot$ kg⁻¹ for a period of 12 weeks had previously been proved in our lab to develop a DN complication characterized by stable hyperglycemia, proteinuria, and morphological evidence. Similar results had also been reported by other researchers.

In the present study, we dissected renal cortex for RT-PCR determination of TGF- β 1 mRNA expression. The medulla was excluded from the analysis since it had been reported that TGF- β 1 mRNA level in the medulla detected by *in situ*

Fig. 5. Effect of Iridoid total Glycoside on Regulating Expression of TGF- β 1 mRNA

The relative amount of TGF- β 1 mRNA level was expressed as normalizing ratio of the densitometric scanning value between TGF- β 1 and β -actin. Data are expressed as means $\pm$ S.D. # p <0.05, diabetic control versus normal control; * p <0.05, ** p <0.01, treatments versus diabetic control.

hybridization did not show any difference from non-diabetic controls,¹⁷⁾ and in another report, using Northern blot analysis of whole kidney RNA, the writer failed to detect an increase of TGF- β 1 mRNA in *db/db* mice versus control. Because the predominant overexpression lies in the glomerular compartment, and the glomeruli comprise <10% of the renal parenchyma, the local increase in glomerular TGF- β 1 was masked by the lack of increase in tubular and medullary TGF- β 1 when whole kidney RNA was examined. TGF- β 1 overexpression had been proved in various experimental diabetes models such as streptozotocin-induced diabetic animals,¹⁸⁾ *db/db* mice,¹⁹⁾ NOD mice,²⁰⁾ and in both diabetes 1 and 2 patients.²¹⁾ A significantly higher protein level was also found in serum of the diabetic control rats in this study. Increasing circulating TGF- β 1 in serum and urine had also been reported closely related to the onset and progression of DN.²²⁾ In another trial carried out in 68 patients with type II diabetes mellitus and a group of 10 healthy subjects serving as control, it was found that both urine and serum TGF- β 1 concentration were elevated and a positive correlation between urine TGF- β 1 level and the progression of renal failure measured by the increase of serum creatinine level was observed.

Fibronectin and laminin are two major ECM expressed and secreted by inherent renal cells. In the diabetes state, both overdeposited in mesangium and tubulointerstitium, which was noted by immunohistochemistry observation in this study and also had been proved by other reports. Overdeposition of ECM that could be caused by higher expression and less degradation led to thickened base membrane, a decreased filtration area but increased permeability resulting from proteinuria.²³⁾ TGF- β 1 had been confirmed to play a pivotal role in the pathogenesis of ECM accumulation in DN. This growth factor exerts its prosclerotic effects by increasing matrix synthesis, decreasing its degradation, and modulating cell-matrix interactions.²⁴⁾ In a recent report, exogenous TGF- β 1 was found to be capable of suppressing proMMP-3, and on the contrary, augmenting proMMP-9 and TIMP-3, which may further suggest its role in ECM reconstitution.²⁵⁾

Chronic hyperglycemia, a necessary prerequisite for the development of DN, leads to the formation of long-lived nonenzymatically glycated proteins that are referred to as AGEs. As a consequence of increased substrate (glucose) availability, AGE accumulate at an accelerated rate in patients with diabetes, where they have been postulated to play a major role in the pathogenesis of the microvascular complications of diabetes. *In vitro* studies have shown that AGEs induced increased ECM production via both TGF- β - and PDGF-dependent mechanisms.^{26–29)} And, according to a recent report, TGF- β 1 may advance fibrosis through activation of the Smad gene family.³⁰⁾ Aminoguanidine, as a positive control in the present study, which had been reported to be a specific inhibitory agent of AGE formation, showed a significant inhibitory effect on serum protein level and glomerular mRNA expression of TGF- β 1, and an apparent effect on preventing overdeposition of fibronectin and laminin. This may cast light on our understanding of the effects and mechanisms of iridoid total glycoside since previously, at a dose of $1 \text{ g} \cdot \text{kg}^{-1}$, it had also proved to be effective in inhibiting the over-deposition of AGEs and overexpression of its main receptor RAGE in the renal cortex of experimental diabetes rats. (Two lower doses of iridoid total glycoside, $0.25 \text{ g} \cdot \text{kg}^{-1}$ and $0.5 \text{ g} \cdot \text{kg}^{-1}$, failed to result in a significantly therapeutic effect, therefore, only the highest dose was included in this paper). As a matter of fact, similar effects on inhibiting TGF- β 1 overexpression and preventing ECM overdeposition between aminoguanidine and iridoid total glycoside were found in the present study. Furthermore, morroniside, a major compound isolated from iridoid total glycoside, had been shown in another research to be effective in protecting endothelial cells from damage by ambient glucose.³¹⁾

In conclusion, iridoid total glycoside from *Cornus* may act as an inhibitory agent of AGE just as aminoguanidine does, thus preventing AGE-induced activation of prosclerotic cytokines such as TGF- β 1 and subsequently preventing overdeposition of ECM. The present study suggests iridoid total glycoside is a potential agent in the prevention and therapy of DN.

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