Beneficial effects of C-peptide on renal morphology in diabetic rats

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The study was undertaken to examine the effects of C-peptide on glomerular volume ($V_{\text{GLOM}}$), mesangial matrix synthesis, and degradation in streptozotocin (STZ)-diabetic rats with poor or moderate glycemic control. Series 1 (poor glycemic control) included groups of healthy rats, hyperglycemic rats, diabetic insulin-treated rats and diabetic C-peptide-treated rats. Series 2 (moderate glycemic control) included groups of healthy rats, diabetic insulin-treated rats, diabetic insulin- and C-peptide-treated rats. After 8 weeks, the left kidney was excised for evaluation of $V_{\text{GLOM}}$ and mesangial matrix area via light microscopy. Mesangial cells were cultured for 48 h and type IV collagen expression and matrix metalloproteinase (MMP)-2 expression were measured by ELISA and RT–PCR. The results indicated that in Series 1, C-peptide administration suppressed the diabetes-induced increase in the $V_{\text{GLOM}}$ and the mesangial matrix area. In Series 2, C-peptide administration resulted in a similar decrease in the $V_{\text{GLOM}}$ and a greater decrease in the mesangial matrix area when compared with insulin therapy alone. Moreover, C-peptide (300 nM) completely inhibited the glucose-induced increase of the collagen IV mRNA expression and protein concentration in mesangial cells cultured in 30 mM glucose medium. MMP-2 mRNA expression was not influenced by C-peptide. In conclusion, C-peptide administration to STZ-diabetic rats for 8 weeks results in the inhibition of diabetes-induced expansion of the mesangial matrix. This effect is independent of the level of glycemic control and results from the inhibition of diabetes-induced excessive formation of mesangial type IV collagen.

Keywords C-peptide; mesangial matrix; type IV collagen; diabetic rat; mesangial cell

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Introduction

Since 1990, several studies in type 1 diabetic patients with incipient nephropathy have revealed the beneficial effects of C-peptide on glomerular hyperfiltration and urinary albumin excretion [1–3]. Studies involving experimental animals have confirmed the protective effects of C-peptide on diabetes-induced glomerular hyperfiltration and renal structural changes [4–6]. The extent to which C-peptide’s beneficial effects depend on the level of metabolic control has not been determined. The main objective of the present study was to elucidate and extend previous findings on the effects of C-peptide on renal morphology under conditions of poor and moderate glycemic control in streptozotocin (STZ)-induced diabetic rats.

The mechanism of C-peptide’s beneficial effects on diabetic nephropathy is still unclear. In diabetic conditions, an imbalance in the synthesis and degradation of extracellular matrix (ECM) components, particularly type IV collagen, by mesangial cells results in the accumulation of mesangial matrix and thickening of the basement membrane within the glomeruli [7,8]. Matrix metalloproteinase (MMP)-2 is the principal type of MMP secreted by mesangial cells and it is the main MMP responsible for the degradation of type IV collagen [9,10]. Therefore, we also investigated the effects of C-peptide on type IV collagen and MMP-2 expression in cultured glomerular mesangial cells of rats to evaluate the possible influence of C-peptide on matrix synthesis and degradation by mesangial cells.

Materials and Methods

In vivo study

Diabetes was induced in male Sprague–Dawley rats (3 months old; initial body weight ~200–250 g) by intravenous injection of STZ (Sigma-Aldrich, St. Louis, USA) at a dosage of 50 mg/kg body weight. The in vivo study began 2 weeks after the STZ injections. Healthy rats of the same age were used as controls.

Pharmacokinetics of C-peptide. Human C-peptide was synthesized by the chemical solid-phase method and TSK column purification with 98.5% purity [11] in the Institute
of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). It was injected subcutaneously at a dosage of 130 nmol/kg in three diabetic rats. The plasma concentrations of human C-peptide in rats were measured by human C-peptide radioimmunoassay kit (China Institute of Atomic Energy, Beijing, China) before injection and 30, 60, 120, and 180 min after injection.

Experimental protocol. Four groups of the rats were used in Series 1 (poor glycemic control), which included (i) normoglycemic control rats (N group, n = 8); (ii) diabetic rats with hyperglycemia (21 mM) and no treatment (HG group, n = 9); (iii) diabetic rats with nearly normal glycemic control (8 mM) with a subcutaneous (sc) daily injection of protamine zinc insulin (Shanghai Biochemistry Pharmaceutical Factory, Shanghai, China) (NG-I group, n = 8); (iv) diabetic rats with hyperglycemia (21 mM) treated twice daily (12 h-interval) with sc injections of human C-peptide at a dosage of 130 nmol/kg (HG-C group, n = 9).

Three groups of rats were used in Series 2 (moderate glycemic control), which included (i) normoglycemic control rats (N group, n = 11); (ii) diabetic rats with moderate glycemic control (11 mM) that were given a daily sc injection of protamine zinc insulin (MG-I group, n = 8); (iii) diabetic rats with moderate glycemic control (11 mM) that were given a daily sc injection of protamine zinc insulin and treated twice daily (12 h-interval) with sc injections of 130 nmol/kg human C-peptide (MG-IC group, n = 10).

The animals in Series 1 and 2 received insulin and C-peptide as appropriate by sc injections for 8 weeks.

Kidney weight and renal morphology. The rat was anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital, and then the left kidney was excised. The kidney was weighed and the kidney-weight (KW) to body-weight (BW) ratio was calculated. A mid-portion coronal slice of 2–3 mm thickness was fixed in 10% neutral formalin, embedded in paraffin, cut into 3-μm thick sections and stained with periodic acid-Schiff reagent. The glomerular volume (V_GLOM) and the ratio of the ECM area to glomerular cross-sectional area (A_ECMA/GLOM) were determined under light microscopy using the IMS Color Image Analyzing System (Shanghai Shen Teng Information Technology Co., Shanghai, China).

In vitro study
Culture of mesangial cells. Glomeruli were isolated from the kidneys of normoglycemic male Sprague–Dawley rats (200 g) by sieving under sterile conditions. The primary culture of mesangial cells was established by plating collagenase-treated glomeruli in RPMI 1640 tissue culture medium (Gibco, Grand Island, USA) containing 20% fetal calf serum, insulin (0.66 U/ml) and antibiotics. Mesangial cells were identified by morphology (stellate-shaped cell bodies with irregular cytoplasmic projections) and their immunofluorescence-staining pattern (positive for desmin; negative for cytokeratin and CD31). All experiments were performed using cells between the 7th and 10th passages.

Experimental protocol. Mesangial cells were plated in 25 cm^2 culture flasks and were deprived of serum for 24 h before use. After the cells reached confluence, the flasks were assigned to one of the following three culture conditions for 48 h: 10 mM glucose (10 mM-G, n = 6), 30 mM glucose (30 mM-G, n = 6) and 30 mM glucose plus 300 nM human C-peptide (30 mM-G/300 nM-C, n = 6). All experiments were performed under serum-free conditions in the absence of insulin.

ELISA of culture media. After 48 h of treatment, cell media were collected. The protein levels of type IV collagen in culture media were measured using the Biokey™ rat collagen Type IV ELISA test kit (LIFEKEY BioMeditech, Monmouth Junction, USA). The ELISA method of measuring protein levels of type IV collagen required that the culture media were freeze-dried and re-diluted into appropriate concentrations as test samples. Test samples and standards were dispensed into the appropriate wells, which were pre-coated with a purified rat collagen type IV antibody, and then incubated for 30 min at 37°C. Then, the incubation mixture was removed. A second purified antibody conjugated to horseradish peroxidase was added into each well. The mixture was incubated for 30 min at 37°C and then removed. A solution of H_2O_2/ tetramethylbenzidine was added and incubated for 15 min at 37°C resulting in color development. The color development was stopped with the addition of 2 N HCl. Absorbance was measured spectrophotometrically at 450 nm. All the assays were performed in duplicate. A standard curve was constructed, and the concentration of rat type IV collagen in each sample was determined. The total amount of rat type IV collagen in the culture medium was then calculated.

MMP-2 activity in the culture media was determined with the Biotrak MMP-2 activity assay system (Amersham Pharmacia Biotech, Little Chalfont, UK). The ELISA method of measuring MMP-2 activities dispensed test samples (culture supernatants) and standards into the appropriate wells that had been pre-coated with anti-MMP-2 antibodies, and then all samples were incubated at 4°C overnight. As a result any MMP-2 present bound to the wells, and the other components of the sample were removed via washing and aspiration. The detection reagents, which consisted of a detection enzyme and a specific chromogenic peptide substrate, were added.
into all wells and these wells were then incubated at 37°C for 1.5 h. Free active MMP-2 activated the detection enzyme, which in turn acted on the substrate to generate a color. The resultant color was detected at 405 nm in a microtiter plate spectrophotometer. All the assays were performed in duplicate. The concentration (ng/ml) of active MMP-2 in a sample was determined by interpolation from a standard curve. The total amount of active MMP-2 in the culture medium was then calculated.

Measurement of gene expression in mesangial cells by RT–PCR. Total RNA of mesangial cells was extracted using Trizol reagent (Gibco). The RNA concentration was measured by spectrophotometric determination at 260 and 280 nm. Each reaction mixture contained 2 μg RNA and was reverse-transcribed into cDNA using 100 pmol of random primer (6mer) (TaKaRa Biotechnology, Dalian, China). Two microliters of first-strand cDNA of type IV collagen or MMP-2 was separately amplified using 3 units of Taq polymerase, 10 mM dNTPs, and 50 pmol of each forward and reverse primer (Shanghai BIOASIA Biotechnology, Shanghai, China). The PCR conditions were as follows: 94°C for 2 min, followed by 29 circles: 94°C for 30 s, 54°C (for type IV collagen) or 56°C (for MMP-2) for 45 s, and 72°C for 1 min. For each gene product, a final extension at 72°C for 6 min was used. Primers for type IV collagen were 5'-cattctttttatgtaagt-3' (sense), 5'-acataacaagaaagaaaaact-3' (antisense), amplification product of 257 bp; for MMP-2, 5'-agatcatacaggtctgag-3' (sense), 5'-atcagcgcattta-3' (antisense), amplification product of 320 bp. β-actin was used as an internal control for loading and reverse transcription efficiency. Its primers were 5'-aactgtgcccatctatggg-3' (sense), 5'-actgtgcccatctatggg-3' (antisense), and the amplification product is 460 bp. The resulting PCR products were electrophoretically fractionated on agarose gels (1.5%) containing 0.5 μg/ml ethidium bromide. The gels were photographed and the intensity of the bands was determined with the GIS 2008 Gel Image Analysis System (Tanon Technology, Shanghai, China). Results were expressed as a ratio of band intensity to the intensity of β-actin.

Statistical analysis
Data are presented as mean ± SEM. Differences between groups were calculated by analysis of variance (ANOVA), followed by Fisher’s least significant difference (LSD) post hoc test. P < 0.05 was considered as statistically significant.

Results

In vivo study
Pharmacokinetics of C-peptide. Before injection, the plasma human C-peptide levels in rats were (0.34 ± 0.15) nmol/l. The plasma C-peptide concentration increased to a peak of (8.80 ± 0.23) nmol/l at 30 min after injection, and then the concentrations declined to 8.16 ± 0.38 nmol/l at 60 min, 6.19 ± 0.53 nmol/l at 120 min, and 0.69 ± 0.57 nmol/l at 3 h after injection.

Effects of C-peptide in Series 1. The average blood glucose level was 22.1 ± 0.7 in the HG-C group and 21.4 ± 0.5 mmol/l in the HG group in Series 1. Glucose levels were higher than those in the NG-I group [(8.7 ± 1.1) mmol/l] and the N group [(5.0 ± 0.3) mmol/l] (P < 0.01) (Fig. 1). The HG-C group had a KW/BW ratio [(4.66 ± 0.24) × 10⁻³] that was lower than that in the HG group [(5.21 ± 0.11) × 10⁻³] (P < 0.05) but higher than those in the NG-I group [(3.35 ± 0.16) × 10⁻³] and the N group [(3.33 ± 0.04) × 10⁻³] (P < 0.01) (Fig. 2).
The $V_{GLOM}$ in HG-C group $[(3.38 \pm 0.24) \times 10^5 \mu m^3]$ was only 50% of that in the HG group $[(6.73 \pm 0.46) \times 10^5 \mu m^3]$ ($P < 0.01$) and similar to those in the NG-I group $[(3.82 \pm 0.34) \times 10^5 \mu m^3]$ and N group $[(3.21 \pm 0.16) \times 10^5 \mu m^3]$. The $AE_{ECM}/AE_{GLOM}$ ratio in the HG-C group $(0.228 \pm 0.005)$ was 31% lower than that of the HG group $(0.329 \pm 0.006)$ ($P < 0.01$) and similar to those in the NG-I group $(0.246 \pm 0.011)$ and the N group $(0.220 \pm 0.006)$ ($P > 0.05$) (Fig. 2).

Effects of C-peptide in Series 2. In Series 2, the average glucose concentrations of the MG-IC group $(11.7 \pm 0.9 \text{ mmol/l})$ and MG-I group $(11.4 \pm 0.9 \text{ mmol/l})$ were similar; both groups’ concentrations were higher than those of the N group $(5.0 \pm 0.2 \text{ mmol/l})$ ($P < 0.01$) (Fig. 1).

The KW/BW ratio in the MG-IC group $[(3.77 \pm 0.15) \times 10^{-3}]$ fell between the values of the KW/BW ratio in the MG-I group $[(3.85 \pm 0.14) \times 10^{-3}]$ and the N group $[(3.39 \pm 0.12) \times 10^{-3}]$, but with no statistical difference (Fig. 3).

The $V_{GLOM}$ in the MG-IC group $(6.15 \pm 0.16) \times 10^5 \mu m^3$ was similar to that of the MG-I group $(6.69 \pm 0.66) \times 10^5 \mu m^3$ ($P > 0.05$), both of which were larger than that of the N group $(4.53 \pm 0.37) \times 10^5 \mu m^3$ ($P < 0.05$ and $P < 0.01$, respectively). The $AE_{ECM}/AE_{GLOM}$ in the MG-IC group $(0.287 \pm 0.004)$ was 22% lower than that of the MG-I group $(0.368 \pm 0.015$ ($P < 0.01$), but it was 26% higher than the N group $(0.228 \pm 0.005$ ($P < 0.01$) (Fig. 3).

In vitro study

Collagen expression and accumulation. Compared with the 10 mM-G culture $(100 \pm 2.0\%)$, the 30 mM-G culture upregulated type IV collagen mRNA transcription $(131 \pm 9.4\%)$ in mesangial cells by 31% ($P < 0.05$), which was fully reversed by C-peptide $(86.3 \pm 7.1\%)$ [Fig. 4(A)]. A higher type IV collagen protein content in the cultured medium appeared in the 30 mM-G culture $[(74.8 \pm 1.3) \text{ ng}]$, compared with the 10 mM-G culture $[(68.5 \pm 2.1) \text{ ng}]$. An addition of 300 nM C-peptide to the 30 mM-G culture completely prevented the rise in type IV collagen protein content of the culture medium $[(67.5 \pm 2.1) \text{ ng}]$ ($P < 0.05$) [Fig. 4(B)].

MMP-2 expression and activity. In the 30 mM-G culture, the MMP-2 mRNA expression in mesangial cells $(90.9 \pm 2.1\%)$ was decreased by 9.1% ($P < 0.05$) compared with the cells grown in 10 mM glucose $(100 \pm 2.8\%)$. C-peptide treatment did not restore the MMP-2 expression level $(91.2 \pm 2.8\%)$ [Fig. 5(A)]. The 30 mM-G culture also reduced active MMP-2 level in culture media $(45.5 \pm 1.9 \text{ ng})$, compared with the 10 mM-G culture $(54.5 \pm 3.4 \text{ ng})$ ($P < 0.05$). The reduction in the active MMP-2 level was not influenced by C-peptide treatment $(40.8 \pm 1.8 \text{ ng})$ [Fig. 5(B)].
Discussion

Several studies on type 1 diabetic patients have shown that C-peptide treatment improves renal function by reducing glomerular hyperfiltration and urinary albumin excretion [1–3]. These results, however, are obtained in the presence of good glycemic control. Subsequent animal studies have shown that the acute or 2-week infusion of C-peptide to STZ-diabetic rats results in a correction of glomerular hyperfiltration and a reduction of albuminuria, even in the absence of insulin treatment [4,5]. Furthermore, recent findings indicate that 4 weeks of C-peptide administration prevent glomerular hyperfiltration, glomerular hypertrophy, and mesangial matrix expansion in STZ-diabetic rats during hyperglycemia [6,12]. In view of these findings, the extent to which the benefits of C-peptide depend on the level of glucose control in type 1 diabetics remains unclear. Consequently, the present study was undertaken to elucidate previous findings with respect to the effects of C-peptide on renal morphology under conditions of varying glycemic control in STZ-induced diabetic rats.

There is substantial homology between human C-peptide and rat C-peptide, both of which have 31 amino acids and have similar efficacy in attenuation of 30 mM glucose-induced vascular dysfunction in a skin chamber granulation tissue model in rats [13]. In order to provide a basis for developing C-peptide as a new drug for clinical use, we chose human C-peptide instead of rat C-peptide in the present study.

It has been found that the supraphysiological levels of human C-peptide are required to prevent vascular dysfunction induced by elevated glucose levels in diabetic rats or in a skin chamber granulation tissue model of non-diabetic rats [13]. Alternatively, only physiological concentrations of rat C-peptide are needed [5,6,13,14]. The greater efficacy for the homologous rat C-peptide versus the human peptide may be related to the differences in amino acid sequence [13]. In the present study, we chose the twice daily dosage of 130 nmol/kg subcutaneously injected human C-peptide. Thereafter, the plasma C-peptide concentration reached the supraphysiological peak levels of ~9 nM at 30 min after injection in rats.

The present data show that under conditions of poor glycemic control (Series 1), 8 weeks of C-peptide treatment significantly decreased the KW to BW ratio, the $V_{\text{GLOM}}$, and $A_{\text{ECM}}/A_{\text{GLOM}}$ ratio. Glomerular hypertrophy in diabetes occurs due to mesangial expansion and basement membrane thickening through ECM accumulation [15,16]. The result of the present study, therefore, indicated that C-peptide prevented glomerular and renal hypertrophy in diabetic rats through diminished glomerular ECM accumulation, which decreased mesangial expansion. However, compared with intensive glycemic control by insulin treatment (NG-I group), C-peptide administration under conditions of poor glycemic control resulted in less marked effects on renal hypertrophy, but showed similar reductions in $V_{\text{GLOM}}$ and mesangial glomerular ratio. One explanation is that the influence of hyperglycemia on kidney restructurization...
may involve other factors than those affected by C-peptide. Good glycemic control with insulin is still essential for preventing diabetic nephropathy. Optimal glycemic control, however, may not be the sole factor in the pathogenesis of diabetes-induced renal abnormalities. In Series 2 (moderate glycemic control), the combined treatment with C-peptide and insulin not only had similar preventive effects on glomerular enlargement and renal hypertrophy compared with insulin-only therapy, but also further decreased glomerular ECM accumulation compared with insulin therapy, demonstrating that the C-peptide effect was uninfluenced by insulin.

However, irrespective of the level of glycemic control, C-peptide exerts more marked preventive effects on mesangial ECM accumulation than on renal hypertrophy. Renal size depends not only on $V_{GLOM}$ but also on renal tubular and interstitial volumes [16]. Our results suggest that the effect of C-peptide on limiting renal hypertrophy in diabetes may rely more on restricting ECM accumulation than on influencing renal tubular and interstitial volumes. We propose that an important target site for C-peptide effects in diabetic nephropathy may be the glomerular mesangial cell.

Both the amount and composition of ECM depend on a delicate balance between the synthesis and degradation of ECM. In diabetic nephropathy, increased mesangial matrix synthesis and inadequate degradation co-exist [17]. Consequently, we examined the influence of C-peptide on matrix synthesis and degradation in rat glomerular mesangial cells. In the in vitro study, C-peptide could prevent glucose-induced type IV collagen accumulation in the culture medium of mesangial cells. This is consistent with the finding of our in vivo study, which showed that...
C-peptide attenuates mesangial ECM accumulation in diabetic nephropathy under poor glycemic control. Additionally, C-peptide prevented the over-expression of type IV collagen mRNA in mesangial cells induced by high glucose concentration, suggesting that C-peptide’s effect on mesangial ECM accumulation may result from its limiting effect on the augmentation of matrix synthesis. This is consistent with a recent finding that C-peptide inhibits Type IV collagen at the transcription level both in glomeruli and in cultured murine podocytes [14]. However, C-peptide did not prevent glucose-induced decreases of MMP-2 activity or its mRNA expression, which suggested that C-peptide had little effect on matrix degradation reduction in diabetic nephropathy. However, the possible effects of C-peptide treatment for longer than 48 h on mesangial cell matrix degradation must be explored.

The urinary albumin excretion rate reflects the renal functional changes of early diabetic nephropathy, but we did not find statistical difference of this parameter between groups. However, as the urinary albumin leakage may be affected by many factors such as diet and exercise, further studies are needed to explore the effects of C-peptide on the urinary excretion under better controlled conditions.

In summary, our study demonstrates that the accumulation of glomerular ECM, which leads to mesangial expansion and glomerular and renal hypertrophy, can be prevented by C-peptide administration for 8 weeks in STZ-induced diabetic rats independent of their level of glycemic control. With moderate glycemic control, more marked attenuation of ECM accumulation in diabetic nephropathy occurs with C-peptide replacement than with insulin therapy alone. C-peptide can diminish type IV collagen over-expression and extracellular type IV collagen accumulation induced in mesangial cells cultured in high glucose conditions but cannot prevent glucose-induced decreases in MMP-2 expression and activity. Thus, the effect of C-peptide on mesangial ECM accumulation in diabetic nephropathy may be mediated via the inhibition of excessive matrix synthesis rather than via stimulation of matrix degradation.

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