Delayed Treatment With Human Umbilical Cord Blood-Derived Stem Cells Attenuates Diabetic Renal Injury

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ABSTRACT

Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease worldwide. Excess accumulation of extracellular matrix and the epithelial-to-mesenchymal transition contribute to renal fibrosis, which is associated with DKD. The present study examined whether delayed treatment with human umbilical cord blood–derived stem cells (hUCB-SC) showed a therapeutic effect on DKD progression. Experimental diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 50 mg/kg) into 6-week-old male Sprague-Dawley rats. Age-matched control rats received an equivalent volume of sodium citrate buffer alone. At 4 weeks after the STZ injection when diabetic renal injury had developed, hUCB-SC were administered (1 × 10^6 cells/rat) through the tail vein. Four weeks after administering the hUCB-SC, rats were sacrificed and we measured indices of DKD, including urinary protein excretion as well as fibronectin, α-smooth muscle actin (α-SMA), and E-cadherin mRNA, and protein expression. Diabetic rats developed significantly increased urinary protein excretion and renal hypertrophy compared to those in control rats. Renal expression of fibronectin and α-SMA mRNA, and protein were increased significantly in diabetic rats compared to those in the controls. E-cadherin protein expression in diabetic kidneys decreased significantly. Intravenously administered hUCB-SC effectively reduced proteinuria, renal fibronectin, and α-SMA up-regulation, as well as renal E-cadherin down-regulation in diabetic rats without a significant effect on blood glucose. Engrafted hUCB-SC in diabetic kidneys were confirmed by human DNA PKcs. The results demonstrated that delayed treatment with hUCB-SC attenuated the progression of diabetic renal injury.
and up-regulated extracellular matrix (ECM) mRNA and protein.\textsuperscript{9,10}

**MATERIALS AND METHODS**

**Human UCB Harvest and Stem Cell Preparation**

hUCB-SC were prepared from UCB with the consent of the mothers according to methods described previously.\textsuperscript{11} Briefly, mononuclear cells were separated from UCB using Ficoll-PaqueTM PLUS (Amersham Biosciences, Uppsala, Sweden). After 5 days of culture, suspended cells were removed and adherent cells cultured further. Surface antigens were analyzed by flow cytometry (Beckman Coulter Epics XL; Miami, Fla, USA) to confirm the SC phenotype.

**Animals**

Diabetic animals were established as described previously.\textsuperscript{10} Briefly, 6-week-old male Spague-Dawley rats weighing 200 g purchased from Charles River Laboratory (Seongnam, South Korea) were randomized into three groups (\(n = 7\)/group); controls (CR), diabetic (DR), and diabetic rats treated with hUCB-SC (DR/SC). Experimental diabetes was induced by intravenous injection of STZ (50 mg/kg in sodium citrate buffer; pH 4.5). CR were injected with an equivalent volume of sodium citrate buffer alone. hUCB-SC (\(1 \times 10^6\) cells/rat) were infused through the tail vein 4 weeks after the STZ injection. The rats did not receive any immunosuppressive agents. DR or DR/SC were injected subcutaneously with insulin (2 U/d/rat, HUMULIN N, Lilly, Indianapolis, IN, USA) to maintain blood glucose levels of 350 to 500 mg/dL.

**Biochemical Analyses**

Biochemical analyses were performed as described previously.\textsuperscript{9} Briefly, plasma glucose was measured with the glucose oxidase method. Plasma and urinary creatinine were measured by the modified Jaffe method. The urinary protein concentrations in supernates were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, Calif, USA). Western Blot Analysis

Kidney tissue was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.\textsuperscript{12} In brief, the renal cortex was lysed and normalized by reprobing the membrane with anti-\(\beta\)-actin antibody (Sigma, St Louis, Mo, USA). Anti-fibronectin (1:2000, Santa Cruz Biotecnology, Santa Cruz, Calif, USA), anti-\(\alpha\)-SMA (1:2000, Santa Cruz Biotecnology), and anti-E-cadherin (1:2000, Santa Cruz Biotecnology) were used as primary antibodies.

**Engraftment of hUCB-SC**

We performed an additional experiment to confirm engraftment of hUCB-SC into the kidney. At 1 day after administering the hUCB-SC, two DR/SC were anesthetized for sacrifice. Rats were perfused with 8% buffered formalin via the abdominal aorta. The excised kidneys embedded in Tissue-Tek OCT compound (Sakura FineTek, Tokyo, Japan) were frozen for subsequent staining with anti-human DNA PKcs antibody (1:200, Abcam, Cambridge, Mass, USA). After washing in phosphate-buffered saline, the sections were incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (1:200, Chemicon, Temecula, Calif, USA), and then mounted by fluid with 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Vector Laboratories, Southfield, Mich, USA). Stained kidneys were observed using confocal laser scanning immunofluorescent microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany).

**Statistical Analyses**

All results are expressed as mean values \(\pm\) standard errors. Statistical comparisons were evaluated using analysis of variance followed by Fishers least significance difference test. The level of significance was set at \(P < .05\).

**RESULTS**

**hUCB-SC Improved Proteinuria in Diabetic Rats**

DR showed increased blood glucose, kidney weight, plasma creatinine, and proteinuria at 8 weeks after inducing diabetes compared to age-matched CR (Table 1). Administering hUCB-SC at 4 weeks after inducing diabetes effectively reduced plasma creatinine and proteinuria to control levels without a significant effect on blood glucose.

**hUCB-SC Suppressed Renal Fibrosis in Diabetic Rats**

Fibronectin mRNA and protein expression increased significantly in diabetic kidneys at 8 weeks after inducing diabetes (Figs 1A and 1D). Fibronectin up-regulation suggested increased ECM synthesis. \(\alpha\)-SMA mRNA and protein expression increased significantly in DR (Figs 1B and 1E), whereas E-cadherin protein expression decreased significantly in diabetic kidneys at 8 weeks after inducing diabetes (Figs 1A and 1D). The decreased expression of ECM and the increased expression of E-cadherin were significantly different in DR (Fig 1C), although E-cadherin mRNA expression was unchanged (Fig 1F), suggesting epithelial-mesenchymal transition (EMT). Delayed treatment with hUCB-SC effectively reduced fibronectin and \(\alpha\)-SMA expression and enhanced E-cadherin expression.
hUCB-SC Were Engrafted in Diabetic Kidneys

Human DNA PKcs were detected in the tubulointerstitium of diabetic kidneys (Figs 2A and 2B) after injured by hUCB-SCs, suggesting that they were engrafted in to the diabetic kidneys.

DISCUSSION

The present study showed that delayed treatment with hUCB-SC attenuated progressive diabetic renal injury including proteinuria, ECM accumulation, and EMT. These results were consistent with previous studies demonstrating that bone marrow-derived stem cells reduce diabetic renal injury in STZ mice.6,7 Although blood glucose values of diabetic mice after injection of bone marrow-derived stem cells were lower than those of diabetic mice.6 hUCB-SC did not influence blood glucose in the present study. It is unclear why we did not observe a hypoglycemic effect of hUCB-SC. One possible reason is that pancreatic β-cell toxicity of STZ was more severe in rats than in mice or that the hUCB-SC dose was not sufficient to restore the pancreatic β-cell toxicity caused by STZ. Although the reason hUCB-SC could not reduce blood glucose remains to be further studied, the renoprotective effects of hUCB-SC

Table 1. Biochemical Analysis

<table>
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<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Kidney Weight (g)</th>
<th>Plasma Glucose (mg/dL)</th>
<th>Plasma Creatinine (mg/dL)</th>
<th>Urinary Protein Excretion (mg/24 h)</th>
</tr>
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<tbody>
<tr>
<td>CR</td>
<td>444 ± 30</td>
<td>1.3 ± 0.1</td>
<td>170 ± 9</td>
<td>1.2 ± 0.1</td>
<td>16 ± 0.3</td>
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<tr>
<td>DR</td>
<td>398 ± 16</td>
<td>1.6 ± 0.1*</td>
<td>349 ± 35*</td>
<td>1.7 ± 0.1*</td>
<td>29 ± 7*</td>
</tr>
<tr>
<td>DR/SC</td>
<td>403 ± 13</td>
<td>1.5 ± 0.0</td>
<td>342 ± 36*</td>
<td>1.4 ± 0.1</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

Results are means ± standard errors of seven rats per group. CR, control rats; DR, diabetic rats; DR/SC, DR with human umbilical cord blood-derived stem cells.

*P < .05 vs CR.

Fig 1. Fibronectin, α-smooth muscle actin (SMA), and E-cadherin protein and mRNA expression. Messenger RNA expression was measured using real-time reverse-transcriptase polymerase chain reaction, and protein expression was measured by Western blotting. (A) Fibronectin protein, (B) α-SMA protein, (C) E-cadherin protein, (D) fibronectin mRNA, (E) α-SMA mRNA, (F) E-cadherin mRNA. Results are means ± standard errors. CR, control rats; DR, diabetic rats, DR/SC, DR with human umbilical cord blood-derived stem cells. *P < .05 vs CR. †P < .05 vs DR.
achieved in the presence of hyperglycemia provide a new therapeutic modality to DKD.

We showed that intravenously administered hUCB-SC were located in the tubulointerstitium, results that agree with previous studies showing engraftment of administered stem cells in the injured tubulointerstitium. The therapeutic mechanism of hUCB-SC remains to be studied. A recent study suggested that bone marrow-derived mesenchymal stem cells show beneficial effects on cisplatin-induced acute kidney injury through secretion of insulin-like growth factor-1.

In conclusion, the present study demonstrated that delayed treatment with hUCB-SC had therapeutic effects on the progression of diabetic renal injury.

REFERENCES