RESEARCH ARTICLE

Early, but not late, treatment with human umbilical cord blood-derived mesenchymal stem cells attenuates cisplatin nephrotoxicity through immunomodulation

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CISPLATIN IS A HIGHLY effective chemotherapeutic agent that is widely used to treat various cancers. However, nephrotoxicity is a major limitation of cisplatin treatment, making it difficult to repeatedly administer effective doses (1, 31). Although various approaches for reducing cisplatin nephrotoxicity have been investigated for several decades, vigorous hydration remains the primary approach for preventing nephrotoxicity (4, 27).

Several recent studies have demonstrated that immunological mechanisms are intimately involved in the pathogenesis of cisplatin nephrotoxicity (19, 20, 28, 29). In particular, T lymphocytes were reported to play a key pathophysiological role in a murine cisplatin nephrotoxicity model (20, 26). Specifically, after cisplatin treatment, T-cell-deficient mice exhibited marked attenuation of renal dysfunction, and adoptive transfer of T cells into immunodeficient mice enhanced cisplatin nephrotoxicity (20). These data suggest that modulating T-cell functions may serve as an effective therapeutic approach for counteracting cisplatin nephrotoxicity. Regulatory T cells (Tregs) are lymphocytes with immunomodulatory properties that inhibit effector T cells, both in vitro and in vivo (24, 34). The adoptive transfer of Tregs has also been shown to reduce renal injury and extend survival time in a rodent model of cisplatin-induced nephrotoxicity (18). Based on these mechanistic insights, immunomodulatory therapies involving the manipulation of immune components have been evaluated in cisplatin-nephrotoxicity models and have shown potential in reducing renal injury (18, 25, 26). However, these strategies have been hampered by certain limitations, including the potential risks associated with direct immune manipulation in patients with cancer.

Mesenchymal stem cells (MSCs) have recently been shown to confer renoprotective effects in animal models of acute kidney injury (AKI), including cisplatin-induced nephrotoxicity (9, 22, 23). MSCs have also been suggested to modulate inflammatory conditions (21, 33, 37). However, the underlying mechanism by which MSCs orchestrate the intrarenal inflammatory micromilieu and promote renoprotection in the context of cisplatin nephrotoxicity is unclear. Moreover, previous studies designed to determine the beneficial effects of MSCs in

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cisplatin nephrotoxicity have employed MSC treatment as a preemptive approach. Thus it remains unknown whether MSC treatment after the development of renal dysfunction can also provide renoprotective and immunomodulatory effects.

The objectives of this study were to investigate the renoprotective effects of early and late MSC treatment and to elucidate the mechanism of MSC treatment in a murine model using human umbilical cord blood (hUCB)-derived MSCs (hUCB-
MATERIALS AND METHODS

Experimental model. C57BL/6 mice (8- to 10-wk-old males) were purchased from Orient Bio (Seongnam, Gyeonggi-do, South Korea). All mice were housed in a specific pathogen-free barrier facility. Animal care and treatment were conducted in compliance with our institutional guidelines, as well as international laws and policies. The Samsung Medical Center Animal Care and Use Committee approved all animal studies.

For early treatment experiments, 22 mg/kg of cisplatin (Sigma-Aldrich, St. Louis, MO) were freshly dissolved in 0.9% saline (1 mg/ml) and were injected subcutaneously into C57BL/6 mice. The early treatment experiments consisted of the following four groups: a cisplatin control group, a group administered early hUCB-MSC treatment by intravenous injection, a group administered early hUCB-MSC treatment by intraperitoneal injection, and a group administered early mouse bone marrow (BM)-derived MSC (BM-MSC) treatment by intraperitoneal injection. Since the hUCB-MSCs were xenogenic, C57BL/6 mouse BM-MSCs were used for comparison as syngeneic MSCs. Mice were administered 1 × 10^6 hUCB-MSCs or mouse BM-MSCs by intraperitoneal injection or intravenous tail vein injection, and the same volume of normal saline was delivered to the cisplatin control group on day 1 after cisplatin injection. Mice were euthanized on day 3 after cisplatin injection.

For late treatment experiments, C57BL/6 mice were injected subcutaneously with 20 mg/kg of cisplatin. Because 22-mg/kg of cisplatin resulted in 20% mortality on day 6 in pilot experiments, the dosage of cisplatin was reduced to 20 mg/kg for the late hUCB-MSC treatment experiments. No mortality was observed until day 6 after cisplatin injection with this dosage. Mice were administered 1 × 10^6 hUCB-MSCs by intraperitoneal injection on day 1 after cisplatin injection in the early hUCB-MSC treatment group, or on day 3 in the late hUCB-MSC treatment group. Mice were euthanized on day 6 postcisplatin injection. In all experiments, each group consisted of 6 to 7 mice.

Renal function was assessed by measuring blood urea nitrogen (BUN; Fujifilm, Bedford, UK) or plasma creatinine (Cr; Arbor Assays, Ann Arbor, MI) levels using a colorimetric kit, according to the manufacturer’s protocol.

Preparation of hUCB-MSCs and mouse BM-MSCs. hUCB-MSCs were isolated and cultivated from hUCB, as previously described (10, 13). The MSCs were positive for human leukocyte antigen (HLA)-A, HLA-B, HLA-C, CD73, CD90, and CD105 but negative for CD14, CD34, CD45, and HLA-DR. hUCB-MSCs were differentiated into osteoblasts, chondrocytes, and adipocytes following exposure to specific stimuli that cause differentiation into the appropriate cell types. hUCB-MSCs were used for all experiments and passages.

C57BL/6 mouse BM-MSCs (CellBiologics, Chicago, IL) were cultured according to the manufacturer’s protocol and used at their fourth passage (P4).

Tissue histology analysis. For histological analysis of tissue samples, kidneys were harvested after exsanguination. Tissue injury was assessed in 4-μm formalin-fixed, paraffin-embedded sections after periodic acid Schiff (PAS) staining. Damaged tubules were identified by the presence of diffuse tubular dilatation, intraluminal casts and/or tubular cell blebbing, and vacuolization and detachment in the cortex and outer medulla. Tubule morphology was examined in 6–10 high-power fields (×400 magnification) per PAS section, in a blinded manner. The number of damaged tubules was divided by the total number of tubules in the same field to determine the percentage of damaged tubules, as described (6).

Immunohistochemical staining for CD45, cleaved caspase-3, and Ki-67. Immunohistochemical testing was performed according to previously described methods (9). Briefly, formalin-fixed kidney sections (4-μm-thick) were deparaffinized with xylene, rehydrated in a graded alcohol series, and then transferred to citrate buffer solution (pH 6.0). Slides were placed in a pressure cooker and heated by microwaving for 10 min to enhance antigen retrieval. After cooling, the kidney sections were immersed in a hydrogen peroxide solution (Dako, Carpinteria, CA) for 30 min to block endogenous peroxidase activity, after which they were incubated overnight at 4°C with serum-free protein block (Dako). The next day, the slides were incubated for 1 h at room temperature with a 1:100 dilution of a rat anti-mouse CD45 monoclonal antibody (mAb; BD PharMingen, San Jose, CA), a 1:200 dilution of a rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA), or a 1:400 dilution of a rabbit anti-mouse Ki-67 mAb (Cell Signaling Technology) (26). As a control, staining was performed in the absence of primary antibody for each immunohistochemical procedure. After being rinsed, the CD45-stained sections were incubated for 10 min at room temperature with a secondary antibody using a DAKO LSAB kit (Dako). This kit consists of a labeled streptavidin-biotin reagent system that includes a biotinylated secondary antibody plus horseradish-labeled streptavidin-biotin reagents. The sections for caspase-3 and Ki-67 staining were incubated with dextran coupled with peroxidase molecules and goat secondary antibody molecules (DAKO EnVision; Dako) for 30 min at room temperature. Subsequently, 3,3′-diaminobenzidine tetrahydrochloride (Dako) was applied to the slides to produce a brown color, and the slides were then counterstained with Mayer’s hematoxylin solution (Dako).

Fig. 1. Early treatment with human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSCs) reduces functional and structural renal injuries in a cisplatin-induced nephrotoxicity model. A: blood urea nitrogen (BUN) levels were significantly lower in the 2 early hUCB-MSC treatment groups and mouse BM-MSC treatment group, regardless of the delivery route or xenogeneic source, than in the cisplatin control group on day 3. Values are expressed as means ± SEs. B: on day 3 after cisplatin injection, kidneys from cisplatin control mice showed tubular dilatation, loss of brush borders, vacuolization, and cast formation, whereas kidneys from early MSC-treated mice showed nearly normal morphology [periodic acid Schiff (PAS) staining, ×200 magnification]. C: tubule damage, reported as mean percentages (±SE), was significantly reduced in the early hUCB-MSC treatment groups compared with the cisplatin control group on day 3 after cisplatin injection. D: cells expressing cleaved caspase-3 (arrow) in cisplatin-injured kidney tissue, visualized via brown staining (×400 magnification). E: percentages of cells expressing cleaved caspase-3 in cisplatin-injured kidney tissue were significantly reduced by early hUCB-MSC treatment on day 3 after cisplatin injection. F: semiquantitative analysis of cleaved caspase-3-positive cells in cisplatin-injured kidneys, expressed as percentages of total cells. G: proportions of PKH-26-labeled hUCB-MSCs were significantly increased in cisplatin-injured kidneys of both hUCB-MSC treatment groups (intravenous and intraperitoneal injection) compared with the kidneys of the normal control group receiving hUCB-MSCs. The proportions of PKH-26-labeled hUCB-MSCs were also significantly higher in the spleens of the 2 hUCB-MSC treatment groups than in the spleens of the normal control group that received hUCB-MSCs. In contrast, no significant differences in hUCB-MSC trafficking into the lung or peritoneum were observed among any of the groups. H: PKH-26-labeled hUCB-MSCs in the kidney, visualized via red staining (×200 magnification). AKI, acute kidney injury; Cisplatin control, cisplatin control group; Cisplatin AKI, cisplatin AKI group; Cisplatin AKI 14d, cisplatin AKI group with hUCB-MSCs IV, early hUCB-MSC treatment group by intravenous injection group; Cisplatin AKI with hUCB-MSCs IP, early hUCB-MSC treatment group by intraperitoneal injection; Cisplatin AKI with mouse BM-MSCs IP, early mouse BM-MSC treatment group by intraperitoneal injection; n = 6 per group. *P < 0.05, compared with the cisplatin control group. †P < 0.001, compared with the cisplatin control group.
To calculate the percentage of CD45-, cleaved caspase-3-, or Ki-67-positive cells in cisplatin-injured kidney samples, the whole fields of slides were scanned and analyzed with a TissueFAXS workstation (TissueGnostics, Vienna, Austria), as described previously (9).

**Immunofluorescence analysis for the detection of PKH-26-labeled hUCB-MSCs.** For immunofluorescence analysis, kidney, lung, spleen, and peritoneum tissue samples harvested after exsanguination were immediately soaked in 25% sucrose solution and frozen in a mold. Frozen tissue sections (6-μm thick) were fixed with chilled 95% ethyl alcohol. After drying at room temperature for 30 min, the slides were washed five times with phosphate-buffered solution. Mounting medium (20 μl) containing 4’,6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories, Peterborough, UK) was then applied to the slides. After the slides were covered with a coverslip, PKH-26-labeled cells (red) were visualized under a fluorescence microscope. The percentage of PKH-26 labeled HUCB-MSCs trafficked into each organ was quantified by counting the number of PKH-26-positive cells and dividing this number by the total number of DAPI-positive cells in 10 randomly chosen different fields.

**Multiplex protein assays.** To examine the intrarenal expression of proinflammatory and anti-inflammatory cytokines after cisplatin injection, the expression levels in whole kidney protein extracts of a panel of cytokines and chemokines were measured using a

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**Fig. 2.** Comparable intrarenal leukocyte infiltration in the cisplatin control and early hUCB-MSC treatment groups. **A:** cells in cisplatin-injured kidney tissue expressing CD45 (arrow), visualized via brown staining (×200 magnification). **B:** semiquantitative analysis of CD45+ cells in cisplatin-injured kidney tissue, expressed as percentages of total cells. **C:** percentages of CD45+ cells in cisplatin-injured kidney tissues did not differ among the cisplatin control group, the 2 early hUCB-MSC treatment, and the early mouse BM-MSC treatment groups on day 3 after cisplatin injection; n = 6 per group.

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Table 1. Intrarenal lymphocytes and macrophages on day 3 after cisplatin injection

<table>
<thead>
<tr>
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<th>Cisplatin Control</th>
<th>hUCB-MSCs Intravenous</th>
<th>hUCB-MSCs Intraperitoneal</th>
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</thead>
<tbody>
<tr>
<td>Total B cells, %</td>
<td>31.4 ± 2.0</td>
<td>27.0 ± 2.0</td>
<td>36.1 ± 2.8</td>
</tr>
<tr>
<td>Total T cells, %</td>
<td>36.2 ± 0.3</td>
<td>37.7 ± 1.2</td>
<td>22.3 ± 1.0</td>
</tr>
<tr>
<td>CD4+ T cells among the total T cells, %</td>
<td>62.1 ± 1.2</td>
<td>62.5 ± 1.3</td>
<td>59.1 ± 1.0</td>
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<tr>
<td>CD8+ T cells among the total T cells, %</td>
<td>24.4 ± 1.8</td>
<td>21.9 ± 0.6</td>
<td>25.7 ± 0.4</td>
</tr>
<tr>
<td>CD4+ CD25+ regulatory T cells, %</td>
<td>1.4 ± 0.2</td>
<td>3.6 ± 0.2*</td>
<td>3.1 ± 0.2*</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.1</td>
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The values shown are the mean percentages (±SE) of gated cells (n = 6 per group). Total B cells, CD19+ B cells among lymphocytes analyzed by forward scatter vs. side scatter gating; total T cells, CD3+ T cells among lymphocytes; CD4+ T cells, CD4+ T cells among the total T cells; CD8+ T cells, CD8+ T cells among the total T cells; CD4+ CD25+ regulatory T cells, CD4 and CD25 double-positive cells among lymphocytes; macrophages, F4/80-positive cells among kidney mononuclear cells; hUCB-MSCs, umbilical cord blood-derived mesenchymal stem cells. *P < 0.05 compared with the cisplatin control group.

MILLIPLEX MAP Mouse Cytokine/Chemokine kit (Luminex, Austin, TX), according to the manufacturer’s instructions. This multiplex, particle-based flow cytometric assay uses anti-cytokine mAbs linked to microspheres with two fluorescent dyes. We designed our assay to detect and quantify the production of cytokines. mAbs linked to microspheres with two fluorescent dyes. We included at least 10,000 gated events.

Flow cytometric analysis. Kidney mononuclear cells (KMNCs) were isolated according to previously described methods (2, 9). Briefly, decapsulated kidneys were immersed in Roswell Park Memorial Institute (RPMI) medium (Mediatech, Manassas, VA) containing 5% fetal bovine serum and then mechanically disrupted using a Stomacher 80 Biomaster apparatus (Seward; Worthing, West Sussex, UK). The samples were then strained, washed, and resuspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), after which cellular suspensions were gently overlaid onto a 72% Percoll layer. After centrifugation at 1,000 g for 30 min at room temperature, the KMNCs were collected at the interface of the 36 and 72% Percoll layers. The KMNCs were then washed twice and counted on a hemocytometer using trypan blue exclusion.

To minimize nonspecific antibody binding, isolated KMNCs were preincubated with an anti-CD16/CD32 Fc receptor-blocking antibody for 10 min. Thereafter, the cells were incubated with anti-mouse CD3, CD4, CD8, CD19, CD25, or F4/80 (BD Bioscience, San Jose, CA) antibodies for 25 min at 4°C, washed with FACS buffer, and fixed in 1% paraformaldehyde solution. Acquisition and analysis of 4-color immunofluorescence staining data were performed using a FACS Calibur instrument (BD Biosciences) and FCS Express 4 Flow Research Edition software (DeNovo Software, Los Angeles, CA), respectively. Each assay included at least 10,000 gated events.

Treg depletion in vivo. Treg depletions were achieved through the intraperitoneal injection of an anti-CD25 mAb (PC61; Bio X Cell, West Lebanon, NH). Briefly, mice were administered 500 μg of anti-CD25 mAb 3 days before and on the day of cisplatin injection and were killed on day 3. CD4+ CD25+ Treg depletion in kidney and spleen samples at the time of death was confirmed by flow cytometry using phycoerythrin-conjugated anti-mouse CD25 and PerCP-conjugated anti-mouse CD4 antibodies.

Statistical analysis. All data are expressed as means ± SE. Group means were compared using the Mann–Whitney test or ANOVA, followed by Newman-Keuls post hoc analysis (using GraphPad Prism, version 5). P < 0.05 was considered statistically significant.
RESULTS

Early treatment with hUCB-MSCs attenuated cisplatin nephrotoxicity. To confirm whether the renoprotective effect of early hUCB-MSC treatment is not affected by the cell delivery route and xenogenic source, changes in renal function were compared in the following four groups after cisplatin injection: the group receiving hUCB-MSCs by intravenous injection, the group receiving hUCB-MSCs by intraperitoneal injection, and the cisplatin control group. Renal function and structural injuries were compared on day 3 after cisplatin injection, at which time all animals with AKI were still alive. Mean BUN levels increased markedly after cisplatin injection and were significantly lower on day 3 in the three treatment groups receiving early MSC treatment compared with the cisplatin control group (Fig. 1A).

The renal histological findings on day 3 in mice with cisplatin-induced nephrotoxicity included focal and severe tubule-cell degenerative changes and luminal casts (Fig. 1B). In the early hUCB-MSC and mouse BM-MSC treatment groups, renal tubule damage (reported as mean percentage) was markedly attenuated (Fig. 1C). In addition, the percentages of cells expressing cleaved caspase-3 (a marker of apoptosis) were significantly reduced in the early hUCB-MSC and BM-MSC treatment groups compared with the cisplatin control group (Fig. 1, D–F). Overall, early treatment with hUCB-MSCs or mouse BM-MSCs showed a renoprotective effect against cisplatin-induced nephrotoxicity, and this effect was not altered by the cell delivery route or MSC source.

To evaluate the organ distribution of the hUCB-MSCs administered by intravenous or intraperitoneal injection, the hUCB-MSCs were labeled with PKH-26 dye and quantified under a fluorescence microscope. The proportion of PKH-26-labeled hUCB-MSCs was significantly higher in the cisplatin-injured kidneys of the hUCB-MSC treatment group compared with the cisplatin control group (which did not receive hUCB-MSCs) and the normal control group (which did receive hUCB-MSCs) (Fig. 1G). Moreover, the proportions of PKH-26-labeled hUCB-MSCs were also significantly higher in the spleens of the two hUCB-MSC treatment groups (intravenous and intraperitoneal injection) compared with the spleens of the normal control group (which received hUCB-MSCs) (Fig. 1G).

Early treatment with hUCB-MSCs showed immunomodulatory effects. We next assessed CD45 expression by immunohistochemical staining to examine leukocyte trafficking into cisplatin-injured kidneys (Fig. 2A). No significant difference in the percentage of CD45-positive leukocytes was observed among the cisplatin control and the early treatment groups with hUCB-MSCs or BM-MSCs (Fig. 2, B and C). We also analyzed T-cell, B-cell, and macrophage populations in the kidneys of cisplatin-injected mice on day 3 postcisplatin injection by flow cytometry. The percentages of total T cells, total B cells, CD4+ and CD8+ T cells, and macrophages on day 3 were comparable between the cisplatin control group and the early hUCB-MSC treatment group on day 1 (Table 1). However, the proportion of intrarenal CD4+ CD25+ Tregs was markedly increased in the early hUCB-MSC and mouse BM-...
MSC treatment groups regardless of the injection route or the source of MSCs, compared with the cisplatin control group (Fig. 3A). In addition, the proportion of CD4<sup>+</sup> CD25<sup>+</sup> Tregs was also increased in spleens from the early hUCB-MSC and mouse BM-MSC treatment groups (Fig. 3B).

On day 3, intrarenal expression of proinflammatory cytokines (MCP-1, IL-6, and TNF-α) was significantly lower in the early hUCB-MSC treatment groups compared with the cisplatin control group; this decrease was observed regardless of the injection route (Fig. 4). In contrast, intrarenal expression of IL-10 and VEGF was higher in the early hUCB-MSC and mouse BM-MSC treatment groups, compared with the cisplatin control group. There was no difference in the expression of IL-2 among the cisplatin and three treatment groups.

In vivo Treg depletion blunted the renoprotective effect of early hUCB-MSC treatment. To further delineate the role of Tregs in the renoprotective and immunomodulatory effects of early treatment with hUCB-MSCs, we investigated whether Treg depletion affected these phenomena. Anti-CD25 mAb treatment can induce Treg depletion with no significant effect on non-Treg cells (6, 18). A dose of 500 μg of anti-CD25 mAb was injected 3 days before and on the day of the cisplatin injections. Rat IgG was used as an isotype-matched negative control. The efficacy of the anti-CD25 mAb treatment was evaluated in kidney and spleen tissue samples harvested on day 3 after the cisplatin injections. Anti-CD25 mAb treatment was found to significantly reduce CD4<sup>+</sup> CD25<sup>+</sup> Treg infiltration (Fig. 5).

On day 3 after the cisplatin injections, the mean BUN levels, percentage of damaged tubules, and percentage of kidney cells expressing cleaved caspase 3 were significantly higher in the anti-CD25 mAb plus hUCB-MSC group than in the isotype control plus hUCB-MSC group (Fig. 6).

Late treatment with hUCB-MSCs did not prevent AKI progression. For the late treatment experiments, we reduced the cisplatin dose to prevent animal death on day 6 posttreatment. The early hUCB-MSC treatment group was also included in this experiment to confirm that renoprotection still occurred after reducing the cisplatin dose and to investigate whether renoprotection was sustained through day 6. On day 6, the mean BUN and plasma Cr levels were significantly lower in the early hUCB-MSC treatment group (Fig. 7, A and B). The renoprotective effect of early hUCB-MSC treatment was sustained until day 6, whereas late treatment with hUCB-MSCs showed no significant renoprotective effect on day 6 (Fig. 7, A and B).

Renal histological examinations of mice with cisplatin-induced nephrotoxicity on day 6 revealed focal tubule cell degenerative changes and luminal casts (Fig. 7C). In the kidneys of the early hUCB-MSC treatment group, the mean percentage of damaged renal tubules was markedly attenuated, whereas late treatment with hUCB-MSCs did not attenuate renal tubule damage (Fig. 7D). The percentage of apoptotic tubule cells expressing cleaved caspase-3 on day 6 was lowest in the early hUCB-MSC treatment group and highest in the cisplatin control group, although no significant differences

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![Figure 5](http://ajprenal.physiology.org/)

**Fig. 5.** Depletion of CD4<sup>+</sup> CD25<sup>+</sup> Tregs in kidney and spleen tissue by treatment with an anti-CD25 monoclonal antibody (mAb). The efficacy of CD4<sup>+</sup> CD25<sup>+</sup> Treg depletion was confirmed by flow cytometry using a phycoerythrin-conjugated anti-mouse CD25 mAb and a PerCP-conjugated anti-mouse CD4 mAb. The percentages of CD4<sup>+</sup> CD25<sup>+</sup> Tregs in kidney and spleen tissue were significantly decreased after anti-mouse CD25 mAb treatment. Isotype plus hUCB-MSCs, group treated with an isotype control (rat IgG) and hUCB-MSCs; anti-CD25 plus hUCB-MSCs (n = 6 per group). *P < 0.05, compared with the isotype control group.
were observed among the three groups on day 6 (Fig. 7, E–G).

The percentage of proliferating tubule cells expressing Ki-67 was significantly higher in the early hUCB-MSC treatment group compared with the cisplatin control group, but late treatment with hUCB-MSCs did not show a significant difference compared with the cisplatin control group (Fig. 7, H–J).

To verify the delivery of hUCB-MSCs into the kidneys of the late treatment group, the proportion of PKH-26 labeled hUCB-MSCs that had reached the kidneys of the late treatment group on day 6 after cisplatin injection (3 days after hUCB-MSC injection) was analyzed by fluorescence microscopy and compared with the proportion reaching the kidneys of the early treatment group on day 3 after cisplatin injection. The trafficking of PKH-26 labeled hUCB-MSCs into the kidneys of mice injected with cisplatin on day 6 was significantly reduced compared with that of the early treatment group on day 3 (Fig. 7K).

Late treatment with hUCB-MSCs did not yield an immunomodulatory effect. On day 6 after cisplatin injection, the percentage of CD45-positive leukocytes (out of the total cells) did not differ significantly among the three groups (Fig. 8). Using flow cytometric analysis, we analyzed T-cell, B-cell, and macrophage populations in the kidneys of mice injected with cisplatin on day 6. The populations of all examined immune cells, including CD4+ CD25+ Tregs, were comparable among the three groups (Table 2).

We next analyzed the intrarenal expression of a panel of cytokines and chemokines (Fig. 9). The early hUCB-MSC treatment group showed significantly lower MCP-1 levels and higher VEGF levels compared with the cisplatin control group. However, the late hUCB-MSC treatment group did not show any significant differences in cytokine production, compared with the cisplatin control group.

**DISCUSSION**

In this study, we demonstrated that early hUCB-MSC treatment increased intrarenal Treg infiltration and affected intrarenal cytokine production during the early injury phase (on day 3 after cisplatin injection) in a murine cisplatin nephrotoxicity model. The renoprotective effect of early hUCB-MSC treatment was blunted after Treg depletion. Furthermore, we investigated whether late treatment with hUCB-MSCs could prevent AKI progression after the development of renal dysfunction. Late treatment with hUCB-MSCs did not alter intrarenal inflammatory components or prevent AKI progression in our murine cisplatin nephrotoxicity model. To our knowledge, this is the first study to demonstrate changes in intrarenal inflammatory conditions (including immune cells) following hUCB-MSC treatment, as well as the first to investigate the renoprotective effects of late hUCB-MSC treatment on cisplatin nephrotoxicity.
Fig. 7. Comparison of the efficacies of early vs. late treatment with hUCB-MSCs to prevent AKI progression following cisplatin-induced nephrotoxicity. Early treatment with hUCB-MSCs during cisplatin-induced nephrotoxicity led to significant improvements in BUN and plasma creatinine (Cr) levels on day 6 (A and B) and reduced the extent of tubule injury (C and D). A–D: these renoprotective effects of early hUCB-MSC therapy were sustained for 6 days after cisplatin administration. In contrast, late treatment with hUCB-MSCs did not prevent the progression of significant functional (A and B) or structural (C and D) renal injury. E–G: the percentage of apoptotic tubule cells expressing cleaved caspase-3 (arrow) was lowest in the early hUCB-MSC treatment group and highest in the cisplatin control group, although no significant differences were observed between the 3 indicated groups. H–J: the percentage of proliferating tubule cells expressing Ki-67 (arrow) was significantly higher in the early hUCB-MSC treatment group compared with the cisplatin control group (magnification, ×400 magnification). K: the delivery of PKH-26-labeled hUCB-MSCs to the kidney was significantly lower in the late hUCB-MSC treatment group compared with the early treatment group. Cisplatin control, cisplatin control group; early hUCB-MSC Tx, early hUCB-MSC treatment group; late hUCB-MSC Tx, late hUCB-MSC treatment group. hUCB-MSCs were administered by intraperitoneal injection on day 1 after cisplatin injection for the early treatment group and on day 3 after cisplatin injection for the late treatment group; n = 6–7 per group. *P < 0.05, compared with the cisplatin control group. †P < 0.05, compared with the early MSC treatment group.
rotoxicity. Our results showed that different therapeutic outcomes occurred following hUCB-MSC administration, depending on the treatment time point.

MSCs show low immunogenicity and have immunomodulatory activity, suppressing inflammatory cytokine production and augmenting the levels of anti-inflammatory cytokines and chemokines (3, 7, 32, 33). Using the immunomodulatory properties of MSCs, previous investigators studied the renoprotective effects of MSCs and showed beneficial effects in various AKI models. Although the mechanism underlying the renoprotection conferred by MSCs has not yet been fully elucidated, Tregs may be involved in immunomodulatory processes during MSC-associated renoprotection, as has been demonstrated in ischemia-reperfusion injury (IRI) models (8, 9).

Previously, we showed that MSC therapy following IRI increased Treg infiltration into kidney tissue (9); Hu et al. (8) also suggested that MSCs ameliorate IRI by inducing Tregs through interactions with splenocytes. We hypothesize that Treg induction may be involved in the mechanism of renoprotection by MSCs during the early injury phase of cisplatin-induced nephrotoxicity. We found that early hUCB-MSC treatment markedly increased intrarenal Treg infiltration. After Treg depletion, the renoprotective effect of early treatment with hUCB-MSCs was blunted, indicating that Treg induction forms part of the mechanism underlying MSC-mediated renoprotection against cisplatin-induced nephrotoxicity.

Tregs have been reported to suppress inflammatory response via multiple mechanisms including modulation of proinflammatory cytokines (16), autocrine adenosine signaling (14), and IL-2-anti-IL-2 complexes (12, 17). Another important therapeutic mechanism of Tregs is their trafficking property to the ongoing inflammatory tissues (35). Distinct expression patterns of chemokine receptors and integrins and their responsiveness contribute to the trafficking of Tregs into inflammatory tissues and selective retention in these tissues (17, 35). Previous studies have also demonstrated that intrinsic Tregs are trafficked into the injured kidney to promote repair (11, 15). We also observed increased Treg trafficking into cisplatin-injured kidney compared with normal kidneys. Moreover, Treg trafficking was augmented via hUCB-MSC treatment. Increased Treg infiltration by MSC treatment may promote immune modulation with IL-10 upregulation.

We analyzed inflammatory conditions in the kidney on day 3 after cisplatin injection, the time at which AKI could first be detected (e.g., by elevation of plasma creatinine or BUN levels) (22, 30) and found that intrarenal inflammatory cytokine expression levels were augmented. We hypothesize that late treatment with MSCs might alter the inflammatory microenvironment to promote a therapeutic effect and prevent AKI pro-

Fig. 8. Similar rates of intrarenal leukocyte infiltration in the cisplatin control group, the early hUCB-MSC treatment group, and the late hUCB-MSC treatment group. A: CD45+ cells (arrow) in cisplatin-injured kidney tissue, visualized by brown staining (×200 magnification). B: semiquantitative analysis of CD45+ cells in cisplatin-injured kidney tissue, expressed as percentages of total cells. C: the percentages of CD45+ cells in cisplatin-injured kidney tissues did not differ between the cisplatin control group, the early hUCB-MSC treatment group, and the late hUCB-MSC treatment group on day 6 after cisplatin injection. Cisplatin control, cisplatin control group; early hUCB-MSC Tx, group administered hUCB-MSCs on day 1 after cisplatin injection; late hUCB-MSC Tx, group administered hUCB-MSCs on day 3 after cisplatin injection; n = 6 per group.
The values shown are the mean percentages (± SE) of gated cells (n = 6 per group). Total B cells, CD19+ B cells among lymphocytes analyzed by forward scatter and side scatter gating; total T cells, CD3+ T cells among lymphocytes; CD4+ T cells, CD4+ T cells among the total T cells; CD8+ T cells, CD8+ T cells among the total T cells; CD4+ CD25+ regulatory T cells, CD4 and CD25 double-positive cells among lymphocytes; macrophages, F4/80+ cells among kidney mononuclear cells; Cisplatin Control, cisplatin control group; Early MSCs Tx, group receiving early treatment with hUCB-MSCs (hUCB-MSCs were administered on day 1 after cisplatin injection); Late MSCs Tx, group receiving late treatment with hUCB-MSCs (hUCB-MSCs were administered by intraperitoneal injection on day 3 after cisplatin injection).

The values shown are the mean percentages (± SE) of gated cells (n = 6 per group). Total B cells, CD19+ B cells among lymphocytes analyzed by forward scatter and side scatter gating; total T cells, CD3+ T cells among lymphocytes; CD4+ T cells, CD4+ T cells among the total T cells; CD8+ T cells, CD8+ T cells among the total T cells; CD4+ CD25+ regulatory T cells, CD4 and CD25 double-positive cells among lymphocytes; macrophages, F4/80+ cells among kidney mononuclear cells; Cisplatin Control, cisplatin control group; Early MSCs Tx, group receiving early treatment with hUCB-MSCs (hUCB-MSCs were administered on day 1 after cisplatin injection); Late MSCs Tx, group receiving late treatment with hUCB-MSCs (hUCB-MSCs were administered by intraperitoneal injection on day 3 after cisplatin injection).

A limitation of this study is that we did not investigate renoprotective or immunomodulatory effects following late treatment with hUCB-MSCs in this study. Specially, no significant differences were observed with respect to intrarenal immune cell populations (including Tregs) or cytokine expression levels between the late hUCB-MSC treatment group and the cisplatin control group.

It is unclear why late treatment with MSCs did not show an immunomodulatory effect. One possibility is that time-dependent participation of immune cells in cisplatin-induced nephrotoxicity may be involved. Liu et al. (20) demonstrated time-dependent T-cell infiltration in a murine cisplatin-induced nephrotoxicity model. In their study, T-cell infiltration into the kidneys could be detected as early as 1 h after cisplatin injection. This infiltration peaked at 12 h and then declined by 24 h (20). It is possible that early treatment with MSCs affects intrarenal-infiltrated immune cells and the inflammatory microenvironment during the initial injury phase to promote renoprotection, whereas late treatment with MSCs does not prevent AKI progression in the late injury phase, after the robust inflammatory process has already occurred, with a decline in the number of immune cells.

In addition, the reduced delivery of MSCs in our late treatment group may have contributed to the diminished therapeutic effects of late MSC treatment. Previous animal studies have demonstrated decreased renal blood flow at 48-72 h after cisplatin injection (5, 36); MSCs were administered at 72 h after cisplatin injection in our late treatment group. Therefore, we propose that decreased renal blood flow after cisplatin injection may have reduced the delivery of MSCs into the injured kidney, consequently diminishing the therapeutic effects of late MSC treatment.

A limitation of this study is that we did not investigate various subsets of immune cells. One previous study evaluated immune cell changes following MSC therapy in an IRI model, and found no significant changes in the numbers of CD4+ or CD8+ T cells. However, significant downregulation of IFN-γ+ proinflammatory chemokine MCP-1 and significantly enhanced VEGF expression in renal tissue compared with the levels observed in the cisplatin control group on day 6. In contrast, late treatment with hUCB-MSCs did not alter cytokine expression compared with the cisplatin control group. Cisplatin control, cisplatin control group; early hUCB-MSC Tx, early hUCB-MSC treatment group; late hUCB-MSC Tx, late hUCB-MSC treatment group. hUCB-MSCs were administered by intraperitoneal injection on day 1 postcisplatin injection for the early-treatment group and on day 3 postcisplatin injection for the late-treatment group; n = 6 to 7 per group. *P < 0.05, compared with the cisplatin control group.

### Table 2. Intrarenal lymphocytes and macrophages on day 6 after cisplatin injection

<table>
<thead>
<tr>
<th></th>
<th>Cisplatin Control</th>
<th>Early MSCs Tx</th>
<th>Late MSCs Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total B cells, %</td>
<td>39.5 ± 2.3</td>
<td>31.1 ± 3.4</td>
<td>35.9 ± 3.0</td>
</tr>
<tr>
<td>Total T cells, %</td>
<td>25.7 ± 2.2</td>
<td>27.0 ± 2.2</td>
<td>30.6 ± 2.7</td>
</tr>
<tr>
<td>CD4+ T cells among the total T cells, %</td>
<td>55.7 ± 1.2</td>
<td>61.5 ± 2.8</td>
<td>55.7 ± 2.6</td>
</tr>
<tr>
<td>CD8+ T cells among the total T cells, %</td>
<td>30.1 ± 0.7</td>
<td>25.8 ± 3.8</td>
<td>29.0 ± 2.5</td>
</tr>
<tr>
<td>CD4+ CD25+ regulatory T cells, %</td>
<td>4.9 ± 0.8</td>
<td>4.8 ± 0.9</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 9. Effects of early vs. late treatment with hUCB-MSCs on intrarenal chemokine and cytokine expression on day 6 after cisplatin injection. Early treatment with hUCB-MSCs resulted in significantly reduced expression of the proinflammatory chemokine MCP-1 and significantly enhanced VEGF expression in renal tissue compared with the levels observed in the cisplatin control group on day 6. In contrast, late treatment with hUCB-MSCs did not alter cytokine expression compared with the cisplatin control group. Cisplatin control, cisplatin control group; early hUCB-MSC Tx, early hUCB-MSC treatment group; late hUCB-MSC Tx, late hUCB-MSC treatment group. hUCB-MSCs were administered by intraperitoneal injection on day 1 postcisplatin injection for the early-treatment group and on day 3 postcisplatin injection for the late-treatment group; n = 6 to 7 per group. *P < 0.05, compared with the cisplatin control group.
MSCs as a novel therapy that could alter the inflammatory response and did not prevent AKI progression. This is in contrast, late hUCB-MSC treatment showed no immunomodulatory effects and might suggest changes in T-cell subtype populations caused by the MSCs.

Conclusions. Our data indicate that early hUCB-MSC treatment could attenuate cisplatin-induced nephrotoxicity in the early injury phase by increasing intrarenal Treg infiltration. In addition, the downregulation of proinflammatory cytokines and upregulation of anti-inflammatory cytokines related to T effector cells was observed (8). In the current study, we compared the effect of fibrate protects from cisplatin-induced ARF. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. We thank Kyung Hee Choi, Ji Woo Kim, and Woo Hyun Lee of the Samsung Biomedical Research Institute (Seoul, Korea), and Se-Hui Kang and Ik-Soon Jang of the Korea Basic Science Institute (Daejeon, Korea) for technical assistance.

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24. Nie CQ, Bernard NJ, Schofield L, Hansen DS. CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the develop-


