VEGF-modified human embryonic stem cell implantation enhances protection against cisplatin-induced acute kidney injury

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Yuan L, Wu MJ, Sun HY, Xiong J, Zhang Y, Liu CY, Fu LL, Liu DM, Liu HQ, Mei CL. VEGF-modified human embryonic mesenchymal stem cell implantation enhances protection against cisplatin-induced acute kidney injury. Am J Physiol Renal Physiol 300: F207–F218, 2011. First published October 13, 2010; doi:10.1152/ajprenal.00073.2010.—The implantation of mesenchymal stem cells (MSC) has been reported as a new technique to restore renal tubular structure and improve renal function in acute kidney injury (AKI). Vascular endothelial growth factor (VEGF) plays an important role in the renoprotective function of MSC. Whether up-regulation of VEGF by a combination of MSC and VEGF gene transfer could enhance the protective effect of MSC in AKI is not clear. We investigated the effects of VEGF-modified human embryonic MSC (VEGF-hMSC) in healing cisplatin-injured renal tubular epithelial cells (TCMK-1) with a coculture system. We found that TCMK-1 viability declined 3 days after cisplatin pretreatment and that coculture with VEGF-hMSC enhanced cell protection via mitogenic and antiapoptotic actions. In addition, administration of VEGF-hMSC in a nude mouse model of cisplatin-induced kidney injury offered better protective effects on renal function, tubular structure, and survival as represented by increased cell proliferation, decreased cellular apoptosis, and improved peritubular capillary density. These data suggest that VEGF-modified hMSC implantation could provide advanced benefits in the protection against AKI by increasing antiapoptosis effects and improving microcirculation and cell proliferation.

vascular endothelial growth factor

ACUTE KIDNEY INJURY (AKI) has long been recognized as a severe and devastating disorder associated with high hospital mortality. The incidence of AKI is ~2,000–3,000 per million per year in the general population, and two-thirds of AKI cases occur in intensive care units (16). Recovery from AKI is usually incomplete, and long-term consequences of AKI can be severe (1, 5, 31, 37). Despite advancements in the research concerning AKI pathogenesis, the current therapeutic option includes supportive measures while patients are waiting for renal function to recover (21, 33), which calls for the development of new and more effective strategies for the treatment of AKI.

The hope of curing AKI has been bolstered by recent developments in stem cell-based therapies. Stem cells, especially mesenchymal stem cells (MSC), have been found to have protective effects in AKI arising from chemical (glycerol and cisplatin) and ischemia-reperfusion (I/R) injuries (15, 25). How stem cells mediate these therapeutic actions remains a subject of controversy. Although some early studies indicated that this protective effect was attributed to the replacement of damaged cells by differentiated stem cells, recent studies have suggested that only a small percentage of repaired tubular cells are stem cell derived (17). More recently, other mechanisms have been suggested to correlate with the therapeutic effect of stem cells in AKI. It was demonstrated that microvesicles derived from human MSC may protect the kidneys from toxic injury by horizontal transfer of mRNA, RNA-dependent apoptosis resistance, and in vitro proliferation (3). Other studies showed that stem cells exert their therapeutic effects in AKI through a paracrine/endocrine mechanism (2, 43, 44) and that MSC produce a variety of cytokines and growth factors (13, 35, 40, 43). Imberti et al. (18), Bi et al. (2), and Togel et al. (44) suggested that the renal protective function comes from insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). It was found that intraperitoneal injection of an MSC-conditioned medium inhibited apoptosis, promoted growth of cisplatin-injured endothelial cells in vitro, and protected mice against AKI (2, 18, 44). Identification and purification of these renoprotective factors would possibly provide new avenues for pharmacological therapy of AKI. Regulation of the paracrine function presents a new method to enhance the therapeutic efficacy of MSC.

VEGF was recently identified as a critical factor in renoprotection by MSC. The effectiveness of MSC was reduced significantly when VEGF was knocked down by small interfering RNA (siRNA) (41, 42). We therefore hypothesized that upregulation of VEGF could strengthen the renal protective effect of MSC. The aim of the present study was to see whether the protective effect of stem cells could be enhanced by increasing VEGF secretion in a cisplatin-induced tubular cell damage model in vitro and in a nude mouse model of cisplatin-induced AKI in vivo.

MATERIALS AND METHODS

Cell Culture and Incubation

A mouse kidney epithelial cell line (TCMK-1, CCL-139) was purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% carbon dioxide. Human embryonic mesenchymal stem cells (hMSC) obtained from embryos aged 4–7 wk were provided by M.-J. Wu. The Committee on Ethics of Biomedicine Research (Second Military Medical University) reviewed and approved all human research protocols, and all donors gave informed consent. The hMSC were grown as described previously (49).
and stored in the Department of Histology and Embryology of the Second Military Medical University.

TCMK-1 cells were seeded at 1.5 × 10^3 cells/cm^2 and incubated 24 h later with DMEM plus 2% FCS alone or in the presence of 10 μM cis-platinum(II)-diamine dichloride (cisplatin; Sigma-Aldrich, St. Louis, MO) for 6 h. The Millicell insert (Millipore, Bedford, MA) co-culture system with a six-well plate was used to study the effects of hMSC on injured tubular cell proliferation. Cisplatin-pretreated TCMK-1 cells were cocultured with hMSC seeded on top of the polycarbonate insert at 1.5 × 10^3 cells/cm^2. After a 3-day coculture, TCMK-1 proliferation and apoptosis were measured.

To study intrarenal localization, hMSC were labeled with a PKH-26 red fluorescence cell linker (Sigma-Aldrich) before infusion into the cisplatin-treated mice.

### Adenoviral Transduction

Ad.CMV-GFP [adenovirus harboring the green fluorescent protein (GFP)-encoding gene], Ad.CMV-VEGF165 (adenovirus carrying human tissue VEGF165 cDNA), and Ad.CMV-con (vacant adenovirus used as control) were kindly provided by Dr. YuDong Zhang (Department of Cardiothoracic Surgery, Affiliated Hospital of Nantong University, Nantong, JS, China).

To select the best multiplicity of infection (MOI) for adenovirus-mediated gene transfer, hMSC were exposed to Ad.CMV-GFP at MOI 20, 50, and 70 for 12 h. Cell viability was observed according to cell morphology by fluorescence microscopy. Cell growth curves drawn to compare the growth between uninfected and infected hMSC. FACS was used to analyze the transfection efficiency of the adopted MOI according to the expression of enhanced GFP (EGFP). The optimal MOI in the subsequent experiments was chosen for both the highest EGFP expression and the best cell viability. VEGF expression in hMSC was detected by RT-PCR and ELISA (R&D Systems, Minneapolis, MN) from day 2 after transduction. The primers used included the following: VEGF: forward primer CCCACTGAGGAGTCCAACAT, reverse primer AAATGCTTTCCTCCGTCTGA; β-actin: forward primer GGCACCCAGCACAAATGAAG, reverse primer CATTGGCGGTGGACGATG.

### Chondrogenic and Adipogenic Differentiation

VEGF- and Ad-modified hMSC (VEGF-hMSC and Ad-hMSC) were seeded onto six-well plates, and differentiation of these cells into chondrocytes and adipocytes was induced at 40–50% confluence. To induce chondrocyte differentiation, cells were cultured with the following chondrogenic differentiation medium: 1.5 × 10^{-4} mg/ml ascorbic acid and 1 ng/ml human recombinant transforming growth factor-β (Sigma). Type II collagen expression was visualized by immunostaining with the mouse monoclonal antibody to human type II collagen (Sigma). To induce adipocyte differentiation, cells were cultured with the following adipogenic differentiation medium: 1 × 10^{-8} mol/l dexamethasone and 1 × 10^{-10} mol/l insulin (Sigma) (49). Two weeks after differentiation, adipocytes were identified by the existence of lipid vesicles stained with Oil Red O (Sigma-Aldrich).

### FACS Analysis

The expression of surface markers on VEGF-hMSC and Ad-hMSC was evaluated by cytofluorimetric analysis. The following anti-human monoclonal antibodies were used: FITC-anti-CD90, FITC-anti-CD34, FITC-anti-CD45, and PE-anti-29 (Biologend). The analysis was performed by a FACSCalibur cytometer (Becton Dickinson). At least 10 events were acquired for each analysis.

Apoptosis was assessed by FITC-annexin V and propidium iodide staining (annexin V-FITC apoptosis detection kit, Bipec Biopharma) according to the manufacturer’s instructions. Cells were analyzed by FACS at 488-nm excitation, green emission for annexin-stained cells and red emission for cells stained with propidium iodide.

### Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) in the medium and in the cellular fraction was measured as an index of lethal membrane injury (necrosis) with an assay kit (CytoTox 96 Non-radioactive Cytotoxicity Assay, Promega) according to the manufacturer’s specifications. Briefly, 3 days after pretreatment with cisplatin, medium was collected and adherence monolayer cells were lysed at 37°C for 45 min. Cell plates were then incubated with the mixed substrate for 30 min in the dark. The reaction was terminated with a stop solution, and absorbance was detected at 490 nm. Cytotoxicity was determined with the following equation: % LDH release = A/(A + B) × 100, where A = LDH release with culture medium — culture medium background and B = LDH release with TCMK-1 — culture medium background.

### In Vivo Experiments

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996). The Committee on Ethics of Biomedicine Research (Second Military Medical University) reviewed and approved all animal research protocols. Male-BALB/c nude mice (6–8 wk old) were purchased from the Chinese Academy of Sciences, Shanghai Laboratory Experimental Animal Center of the Second Military Medical University. All nude mice were allowed free access to standard laboratory chow and tap water, housed in a constant-temperature room with a 12:12-h light-dark cycle, and fed a standard diet. AKI was induced by intravenous tail injection of cisplatin (18 mg/kg). Twenty-four hours after cisplatin administration, nude mice were divided into four groups and received intravenous tail injections as follows: group 1, saline (500 μl; n = 6); group 2, Ad.CMV-VEGF165-transfected hMSC (VEGF-hMSC) (5 × 10^5 cells/500 μl; n = 6); group 3, Ad.CMV-con-transfected hMSC (Ad-hMSC) (5 × 10^5 cells/500 μl; n = 6); and group 4, hMSC (5 × 10^5 cells/500 μl; n = 6). Control animals (n = 6) did not receive cisplatin injections.

To trace hMSC in vivo, nude mice in groups 2 (n = 3) and 4 (n = 3) were administered intravenous injection of PKH-26-labeled stem cells (5 × 10^5 cells/500 μl).

Renal function was assessed in terms of serum creatinine (SCR) and blood urea nitrogen (BUN) determined by enzymatic colorimetric assay at baseline and 4 days after cisplatin injection. Kidneys were harvested for histological analysis.

### Renal Morphology

Kidney samples were immersed in 4% neutral buffered formaldehyde, fixed for 72 h, sliced into 2-μm sections, and stained with hematoxylin and eosin (H & E). Luminal hyaline casts and cell loss (denudation of tubular basement membrane) were assessed in at least 28 nonoverlapping high-power fields (HPF, ×400)/section (n = 6 mice for each group, 2 sections/mouse). The number of casts and tubular profiles showing necrosis was recorded in a single-blind manner.

For PKH-26-labeled MSC quantification, samples were frozen immediately in liquid nitrogen, embedded in OCT compound, sliced into 5-μm sections, fixed in acetone (10 min), and incubated (30 min) with FITC-labeled wheat germ agglutinin (WGA Lectin; Vector Laboratories, Burlingame, CA). Nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma-Aldrich).

PKH-26-positive cells were counted in six frozen renal sections per mouse (n = 3 mice). Data are expressed as the number of PKH-26-positive cells per renal section.
Immunofluorescence and Immunohistochemistry

To determine the number of proliferating tubular cells, the expression of cell and tissue proliferating cell nuclear antigen (PCNA) was detected. TCMK-1 grown on coverslips were fixed with cool methanol and blocked with goat serum for 30 min. Cells were incubated with an anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and then exposed to tetramethylrhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG (Santa Cruz Biotechnology) for 1 h in the dark. Nuclei were stained with DAPI. Negative controls were incubated with PBS instead of the primary antibodies. The fluorescence intensity of PCNA was then estimated by an image analyzer (Image-Pro Plus 6.0). For each slide cells in five randomly chosen fields were observed, and the values of the average optical density (AOD) were measured under the same magnification (HPF, ×400) with a color image analysis system. Five slides were counted in each group.

Paraffin-embedded kidney sections were deparaffinized with xylene and rehydrated in an alcohol series and water. After incubation with goat serum, slides were stained for PCNA and CD34. Nuclei were visualized by counterstaining with Harris hematoxylin. Scoring for PCNA-positive cells was carried out by counting the number of positive nuclei in the renal cortex in 10 HPF (×400)/section (n = 6 mice for each group, 2 sections/mouse). The area percentage of stained microvessel was determined with Motic Images Advanced 3.2 software (Motic China Group). Ten HPF (×400) per section from the cortex were analyzed from each study group and the controls (n = 6 mice for each group, 2 sections/mouse).

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Label Staining

Apoptotic scores were obtained with a terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (AP, Roche Laboratories, Indianapolis, IN). Accordingly, kidney sections were deparaffinized, rehydrated, digested with proteinase K, and labeled with a TUNEL reaction mixture for 60 min at 37°C. TUNEL-positive apoptotic

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**Fig. 1.** Ability of adenoviral vector to infect human embryonic mesenchymal stem cells (hMSC). A: representative image of % of enhanced green fluorescent protein (EGFP) expression in hMSC analyzed by fluorescence microscopy. hMSC were transduced with EGFP adenovirus at multiplicity of infection (MOI) 20, 50, and 70. B: the appropriate MOI was found to be 50, with higher infective efficiency (n = 4, *P < 0.01 vs. MOI 50 and MOI 70; ΔP > 0.05 vs. MOI 70). C: EGFP-positive hMSC maintained fibroblast shapes at MOI 50. D: transduction with EGFP adenovirus at MOI 50 (GFP-hMSC) had no significant effect on hMSC cell growth curve compared with regular hMSC. x-Axis, days after transduction.
tubular epithelial cells were counted in 10 HPF (×400) per section in the cortex (n = 6 mice for each group, 2 sections/mouse).

Statistical Analyses

Data were analyzed by standard statistical methods and analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparisons or the nonparametric Kruskal-Wallis test. Group data are expressed as means ± SD, and P < 0.05 was considered statistically significant. Kaplan-Meier statistics were used for survival analyses.

RESULTS

In Vitro Study

Adenoviral vector transfection of hMSC. The ability of the adenoviral vector to infect hMSC was tested with an Ad-EGFP vector. The cytopathic effect was obvious in hMSC at MOI 100. MSC became round shaped and detached from the wall in 3 days, and <50% of the population survived after a week. hMSC were then infected with Ad-EGFP at MOI 20, 50, and 70. The transduction efficiency, as determined by the percentage of EGFP-positive cells, was 36.13 ± 3.04% at MOI 20, 70.29 ± 3.07% at MOI 50, and 73.63 ± 3.04% at MOI 70 (Fig. 1, A and B). The growth rate of hMSC infected with Ad-EGFP at MOI 50 was similar to that of uninfected control hMSC, and morphologically hMSC remained a homogeneous population of fibroblast-shaped cells (Fig. 1, C and D). Therefore, adenoviral vectors were used to infect hMSC at MOI 50 in the subsequent experiments because of high efficiency and low toxicity.

Characterization of VEGF-modified hMSC. RT-PCR analysis showed that VEGF mRNA was highly expressed 48 h after hMSC transduction (Fig. 2A). VEGF levels in the culture medium were measured by ELISA for 2 wk. It was found that the peak level appeared at day 6 after transduction. At day 4 after transduction, the VEGF level in the medium of the VEGF-hMSC increased significantly, about four times that of the uninfected hMSC (Fig. 2B). To reach a maximal expression of VEGF in vivo, hMSC were injected into the mice 3 days after transduction.

Differentiation and surface markers of VEGF- and Ad-hMSC. To evaluate the multipotent potential of VEGF-hMSC and Ad-hMSC, both types of hMSC were differentially induced into chondrocytes and adipocytes, as demonstrated by type II collagen immunostaining and Oil Red O staining, respectively (n = 3; Fig. 3A).

Flow cytometry analysis showed that both VEGF-hMSC and Ad-hMSC were positive for mesenchymal markers CD29 and CD90 but negative for markers of hematopoietic lineages CD34 and CD45 (Fig. 3B).

In vitro study with a coculture system. The ability of hMSC in every coculture group to induce regeneration of cisplatin-impaired tubular cells was investigated with a coculture system in which cells established indirect contact by secreting factors through the membrane of the coculture system. Three days after pretreatment with cisplatin, the growth rate of TCMK-1 decreased significantly compared with that of normal TCMK-1 (P < 0.01), and this growth-inhibitory effect was attenuated after coculture with hMSC, especially with VEGF-hMSC (P < 0.01 vs. cisplatin + TCMK-1 + Ad-hMSC, cisplatin + TCMK-1 + hMSC; Fig. 4A). The number of TCMK-1 cocultured with VEGF-hMSC was 2 times and 1.5 times those for cisplatin pretreatment alone and coculture with Ad-hMSC/hMSC, respectively.

TCMK-1 cells showed a weak positive staining intensity for PCNA 3 days after cisplatin pretreatment alone (P < 0.01 vs. normal control TCMK-1). After coculture with hMSC, cisplatin-pretreated TCMK-1 showed a stronger fluorescence intensity of PCNA compared with TCMK-1 pretreated with cisplatin alone (P < 0.01). The mean fluorescence gray scale value of PCNA increased significantly in TCMK-1 cocultured with VEGF-hMSC compared with addition of Ad-hMSC and hMSC (P < 0.05), to a level similar to that of normal control TCMK-1 (P > 0.05; Fig. 4, B and C).

The percentage of apoptotic TCMK-1 labeled with annexin-FITC but not with propidium iodide increased obviously 3 days after cisplatin pretreatment alone (P < 0.01 vs. normal control cells). TCMK-1 apoptosis was inhibited by coculture with hMSC (P < 0.01 vs. TCMK-1 pretreated with cisplatin alone). The percentage of apoptotic TCMK-1 reduced by more than 35% and 40% in coculture with hMSC and Ad-hMSC, respectively. However, VEGF-hMSC intensified the protective effect, with the apoptosis percentage dropping to 5.76% (P < 0.01 vs. cisplatin + TCMK-1 + Ad-hMSC and cisplatin + TCMK-1 + hMSC; Fig. 5B).

Cisplatin-induced TCMK-1 injury caused significant and comparable lethal cell injury as represented by 61% LDH release. Coculture with VEGF-hMSC, Ad-hMSC, or hMSC decreased LDH release in TCMK-1 caused by cisplatin (P < 0.01). After 3-day cisplatin pretreatment, TCMK-1 coculture with VEGF-hMSC showed LDH release levels that were half
of those seen when TCMK-1 cells were pretreated with cisplatin alone (Fig. 5C).

An overview of the above results indicates that coculture with VEGF-hMSC enhanced the ability for proliferation and antiapoptosis activity in TCMK-1.

**In Vivo Study**

**Protection of VEGF-hMSC on cisplatin-induced renal injury.** To see whether hMSC could increase its protective effect on AKI through VEGF modification, nude mice were divided into four groups according to the type of hMSC injected via the tail vein 24 h after cisplatin administration. Renal function and tubular structure were impaired in the saline group 4 days after cisplatin administration. hMSC and Ad-hMSC exhibited a renoprotective effect, as reflected by lower BUN and SCr values compared with the saline group ($P < 0.01$). In addition, upregulation of VEGF in hMSC increased the protective effect of hMSC on BUN and SCr ($P < 0.01$ versus Ad-hMSC and hMSC groups; Fig. 6C). The Ad-hMSC and hMSC engraftment decreased renal morphological injury, as indicated by fewer necrotic tubules and casts compared with saline injection ($P < 0.01$). However, VEGF-hMSC further resulted in a more renoprotective effect, as represented by less severe tubular injury ($P < 0.01$ vs. Ad-hMSC and hMSC groups; Fig. 6B).

The engraftment capacity of hMSC in the kidney was evaluated by the presence of PKH-26-labeled cells in renal parenchyma 4 days after cisplatin administration. There was no significant difference in the number of PKH-26-positive cells between the VEGF-hMSC and hMSC groups (1.63 ± 0.68 and...
A certain amount of hMSC was detected in lung, liver, and spleen tissues of the nude mice.

Effects of VEGF-hMSC in reducing renal cell apoptosis and promoting renal cell regeneration after cisplatin-induced renal injury. Regeneration and apoptosis were increased in the kidney 4 days after AKI as represented by elevation of the PCNA and TUNEL staining indexes ($P < 0.01$ vs. normal control group). In all hMSC groups, the number of PCNA-positive cells increased and that of TUNEL-positive cells decreased in renal sections compared with the saline group ($P < 0.01$, Fig. 7, B and C). In the VEGF-hMSC group, the therapeutic effect of regeneration and antiapoptosis was most obvious, as represented by a higher score of PCNA and a lower score of TUNEL staining ($P < 0.01$ vs. Ad-hMSC and hMSC groups; Fig. 7, B and C).
tumors were found in the body organs including the kidney. and hMSC groups were killed 2 mo after cell injection, and no in the saline group was only 20%. and Ad-hMSC were still alive, while the survival rate of mice after AKI 60% and 40% of mice injected with VEGF-hMSC significantly longer than mice infused with saline. At Fig. 9. Mice infused with hMSC 1 day after cisplatin survived survival curves of AKI nude mice in all groups are shown in percentage of peritubular capillaries (P

Effects of VEGF-hMSC in increasing microvessel density after cisplatin-induced renal injury. Functional and structural abnormalities in the renal microvasculature are important factors contributing to the pathophysiology of AKI. With CD34 immunostaining, renal capillary density was determined 4 days after cisplatin-induced AKI. The area percentage of positive staining was obtained. The area percentage of peritubular capillaries shrank in all cisplatin groups compared with that observed in normal nude mice (P < 0.01). The area percentage of peritubular capillaries was higher in the kidney of VEGF-hMSC, Ad-hMSC, and hMSC groups (P < 0.05 vs. saline group). VEGF-hMSC therapy significantly restored the area percentage of peritubular capillaries (P < 0.01 vs. Ad-hMSC and hMSC; Fig. 8B).

Effects of VEGF-hMSC in reducing mortality of AKI mice without causing tumor formation 2 mo after injection. The survival curves of AKI nude mice in all groups are shown in Fig. 9. Mice infused with hMSC 1 day after cisplatin survived significantly longer than mice infused with saline. At day 9 after AKI 60% and 40% of mice injected with VEGF-hMSC and Ad-hMSC were still alive, while the survival rate of mice in the saline group was only 20%.

The surviving nude mice of the VEGF-hMSC, Ad-hMSC, and hMSC groups were killed 2 mo after cell injection, and no tumors were found in the body organs including the kidney.

DISCUSSION

This study demonstrated that transduction of hMSC with the VEGF gene improved their therapeutic benefits in healing cisplatin-induced renal epithelial injury. VEGF-modified hMSC enhanced the renoprotective activity in the AKI nude mice model compared with regular hMSC and vacant adenovirus-infected hMSC.

MSC can be separated from embryo and adult specimens. MSC derived from adult bone marrow are used most commonly in stem cell therapy. Compared with bone marrow-derived MSC, embryo-derived MSC have greater expansion and differentiation potentials. Immunologically, histocompatibility antigens are less intensively expressed in embryo-derived cells of early gestation terms than in adult cells (32, 38, 47). Embryoiderived MSC can be frozen in the laboratory and amplified immediately to meet the requirement for treatment of AKI. However, there have been few studies using human embryo-derived MSC for the treatment of AKI. The hMSC used in this study were isolated from 4- to 6-wk-old embryos from volunteers who underwent pregnancy termination with RU-486, an antiprogesterone compound (49). hMSC can give rise to bone, cartilage, fat, muscle, and neuron tissues. Confluent hMSC are fibroblast-like. Cisplatin nephropathy is usually chosen as an AKI model because of its simplicity and reproducibility. In addition, the pathology and the recovery phase are comparable with those in humans. Finally, a common cause of AKI such as volume depletion is replicated in this model (14).

The hMSC separated in our laboratory have been verified to possess differentiation potential and express relative specific antigens of MSC as previously described (49). FACS results of the present study showed that Ad-hMSC and VEGF-hMSC expressed not hematopoietic lineage markers but mesenchymal cell markers and that both Ad- and VEGF-hMSC were able to differentiate into chondrocytes and adipocytes, suggesting that gene transfer did not alter the MSC characteristics in hMSC.

The paracrine/endocrine mechanism is an important consideration in stem cell therapy. Many beneficial growth factors have been investigated by siRNA blocking studies, including IGF-1, angiopoietin-1 (Ang1), keratinocyte growth factor (KGF), HGF, and VEGF (7, 18, 22, 26, 27, 39). Knockdown of VEGF in stem cells significantly decreased the therapeutic effect of these cells in cardiac ischemia and in brain injury in premature infants (26, 39).
In an ischemic AKI rat model, knockdown of VEGF by siRNA also reduced the therapeutic effect of MSC (41, 42). In these studies, the VEGF level of MSC was knocked down by 60% by siRNA. Compared with that from wild MSC, the medium from VEGF-knockdown MSC decreased the proliferative effect in rat proximal tubular cells in vitro. Adding 10 ng/ml VEGF restored the proliferative activity of the medium from VEGF-knockdown MSC. Rats treated with VEGF-knockdown MSC had a higher mortality and a slower recovery of renal function after AKI. These data clearly demonstrate that VEGF secreted by MSC plays an important renoprotective role in AKI when stem cell treatment is used.

VEGF, a potent effector of vascular development, is normally expressed in the mesenchymal tissue during development and is inducible by hypoxia (8, 9, 30). VEGF can induce proliferation and an antiapoptotic response in renal...
tubular epithelial cells and exerts proangiogenic effects in AKI as well. Renal ischemic injury damage can be ameliorated by treatment with VEGF immediately following injury (23, 34, 46).

A VEGF increase in MSC can enhance the therapeutic effect of stem cells in different diseases (4, 19, 29, 45, 48, 50). VEGF165 has been used to enhance the function of MSC and endothelial progenitor cells (EPC) for stem cell therapy in heart disease (19, 50). Kallikrein-modified MSC in the kidney provide advanced benefits in protection against ischemia-induced kidney injury. Increased VEGF secretion from kallikrein-modified MSC as observed in the present study may also be renoprotective (12). In addition, VEGF receptor (VEGFR)-2 expression is increased in peritubular capillaries and also in some tubular epithelial cells after AKI such as I/R injury (20). Therefore, VEGF upregulation may be a new way to strengthen the therapeutic effect of stem cells in AKI. We combined hMSC with VEGF165 gene transfer to enhance the curative effect of hMSC on renal tubular epithelial impairment in vitro and vivo.

In the present study, we used a model of cisplatin-induced toxicity in proximal tubular cells to test the enhanced reparative capacity of VEGF-modified hMSC with the Millicell insert.
coculture system and found that all hMSC had proliferation effects on injured TCMK-1 even though they were separated by a porous membrane. This finding is consistent with the previous study indicating that soluble factors secreted by MSC accounted for increased injured tubular cell proliferation (18). Furthermore, we found in the in vitro experiment that directly increasing VEGF expression in hMSC by gene transduction could strengthen the antiapoptotic and proliferation effects of these cells. We also found that nude mice treated with VEGF-modified MSC had lower mortality and better renal function after AKI.

It has been reported previously that stem cells protect the kidney against toxic injury by secreting factors that limit apoptosis and enhance proliferation of endogenous tubular cells (2, 28), even though the surviving tubular epithelial cells have been shown as the predominant regenerative source (17, 24). Furthermore, VEGF itself can also mediate mitogenic and antiapoptotic actions on renal tubular epithelial cells (23, 45). The antiapoptotic and mitogenic properties of stem cells found in our study are in agreement with previous results (12, 28), and at the same time we also found that VEGF upregulation in hMSC can strengthen the therapeutic effect.

In AKI, there is a predominance of vasoconstrictive activity due to an imbalance of vasoactive substances. Area reduction and endothelial destruction of peritubular capillaries were reported in an AKI model (11, 28). VEGF is reported to be a maintenance factor for endothelial cells and can also mediate kidney endothelium-dependent vasodilatation in addition to stimulating proliferation and promoting survival of renal epithelial cells (11, 18). We calculated microvessel density in all groups 4 days after AKI and found that the peritubular microvessel density was reduced. The level of shrunken peritubular microvessel density was elevated after hMSC treatment. The most obvious elevation was seen in the VEGF-modified hMSC treatment group compared with the other hMSC groups and the untreated group. Increasing VEGF secretion in hMSC may lead to better endothelial cell protection, preserve renal microcirculation, and ultimately improve renal function.

The location of hMSC in the kidney of AKI mice was observed in VEGF-hMSC and hMSC. There was no significant difference between the two groups. Previous studies (18, 28) reported that very few MSC were found in the kidney of AKI mice 2 days after MSC infusion. In our study, we also confirmed that internal cells were the main source of renal regeneration and that only small numbers of hMSC existed in the kidney. In addition, large numbers of hMSC were observed in other tissues from AKI mice such as the liver, lungs, and spleen between 2 h and 4 days of hMSC infusion. Nutritional factors secreted by hMSC from the kidney may have contributed to renovation of impaired tubular epithelial cells by blood circulation. These factors include EGF, HGF, IGF-I, bone morpho-

![Fig. 8. Assessment of microvessel density in renal cortex of mice 4 days after cisplatin-induced AKI. A: representative images of CD34 staining in renal sections. Original magnification, ×400. B: quantification of vascular area per visual field in the renal cortex. Original magnification, ×400. ΔP < 0.01 vs. Ad-hMSC and hMSC groups; ○P < 0.01 vs. control group and P < 0.05 vs. VEGF-hMSC, Ad-hMSC, and hMSC groups (n = 6). Data are represented as means ± SD.](http://ajprenal.physiology.org/)

![Fig. 9. VEGF-hMSC treatment prolongs survival in mice with AKI. ΔP < 0.01 vs. Ad-hMSC group; ○P < 0.01 vs. saline group.](http://ajprenal.physiology.org/)
gene therapy would prove to be an optimized approach for the reasons, this combined strategy of cell transplantation with hMSC treatment did not induce tumor formation. For the above and protective effects on peritubular capillaries; and protective effect of stem cells by antiapoptosis, proliferation, embryos had a protective effect on tubular injury both in vitro and vivo; 2) VEGF-modified hMSC can strengthen the reno-protection effect of stem cells by antiapoptosis, proliferation, and protective effects on peritubular capillaries; and 3) VEGF- hMSC treatment did not induce tumor formation. For the above reasons, this combined strategy of cell transplantation with gene therapy would prove to be an optimized approach for the treatment of AKI.

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DISCLOSURES
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