Impact of Ex Vivo Administration of Mesenchymal Stem Cells on the Function of Kidney Grafts From Cardiac Death Donors in Rat

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ABSTRACT

Background. Mesenchymal stem cells (MSCs) have been applied to the treatment of various diseases, and MSC administration in marginal donor grafts may help avoid the ischemia–reperfusion injury associated with solid organ transplants. Given the reports of side effects after intravenous MSC administration, local MSC administration to the target organ might be a better approach. We administered adipose tissue–derived MSCs (AT-MSCs) ex vivo to donor rat kidneys obtained after cardiac death (CD).

Methods. Using male Lewis rats (8–10 weeks), and a marginal transplant model of 1 hr CD plus 1 hr sub-normothermic ET-Kyoto solution preservation were conducted. AT-MSCs obtained from double-reporter (luciferase–LacZ) transgenic Lewis rats were injected either systemically (1.0 × 10⁶ cells/0.5 mL) to bilaterally nephrectomized recipient rats that had received a marginal kidney graft (n = 6), or locally via the renal artery (500 μL ET-Kyoto solution containing the same number of AT-MSCs) to marginal kidney grafts, which were then preserved (1 hour; 22°C) before being transplanted into bilaterally nephrectomized recipient rats (n = 8). Serum was collected to assess the therapeutic effects of AT-MSC administration, and the recipients of rats surviving to Day 14 were separately evaluated histopathologically. Follow-up was by in vivo imaging and histological LacZ staining, and tumor formation was evaluated in MSC-injected rats at 3 months.

Results. Systemic injection of MSC did not improve recipient survival. In vivo imaging showed MSCs trapped in the lung that later became undetectable. Ex vivo injection of MSCs did show a benefit without adverse effects. At Day 14 after RTx, 75% of the rats in the AT-MSC–injected group (MSC [+]) had survived, whereas 50% of the rats in the AT-MSC–non-injected group (MSC [−]) had died. Renal function in the MSC(+) group was improved compared with that in the MSC(−) group at Day 4. LacZ staining revealed AT-MSCs attached to the renal tubules at 24 hours after RTx that later became undetectable. Histopathologic examination showed little difference in fibrosis between the groups at Day 14. No teratomas or other abnormalities were seen at 3 months.

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Kidney transplantation is the preferred treatment for end-stage renal disease [1]. However, the annual increase in the number of new patients with end-stage renal disease who need a kidney transplant and the widening gap between the demand for and supply of donor kidneys have led to a progressive worldwide shortage of donor organs [2]. Despite the increased use of organs from marginal donors, waiting lists and deaths while awaiting transplantation have increased. Marginal kidney transplantations are currently limited by inferior outcomes and higher costs [3,4]. Recently, to expand the donor pool, a system of organ donation after cardiac death (CD) has been started in many countries [5–7]. However, compared with kidneys obtained from living or brain-dead donors, those from CD donors are significantly more prone to ischemia–reperfusion injury, which may account for the higher rates of both primary nonfunction and delayed graft function [8,9]. The presence of delayed graft function increases the risk of acute rejection and doubles the rate of graft loss within 5 years [10,11].

Mesenchymal stem cells (MSCs) have been studied extensively and applied therapeutically for organ transplantation [12], and there is a potential clinical application for marginal donor grafts to prevent the ischemia–reperfusion injury associated with solid organ transplants. MSCs are multipotent progenitor cells that are capable of proliferating and differentiating into many different cell lines [13,14]. Adipose tissue is a particularly appealing source of MSCs because it is abundant and easy to procure through minimally invasive procedures [15]. Currently, a large number of MSC-based experimental and clinical trials are being conducted worldwide for the treatment of various diseases [16–22]. Recent reports, however, have documented cases of lethal pulmonary thromboembolism in patients after intravenous administration of adipose tissue–derived MSCs (AT-MSCs) [23–25]. Tissue engineering approaches that allow engraftment of MSCs by local administration might reduce the likelihood of MSC-associated thromboembolism following transplantation [26]. Therefore, in the organ transplant setting, local administration of MSCs to the target organ might be an ideal approach because the donor organ can be perfused ex vivo [27].

We previously developed a sub-normothermic (below room temperature) preservation method utilizing extracellular-type Kyoto solution (ET-K) for the short-term storage of marginal kidneys [28]. Additionally, it was suggested that it might be possible to predict a graft’s function in a CD donor kidney using noninvasive magnetic resonance imaging (MRI) in rats [29]. Therefore, using a marginal transplant model of 1 hour CD plus 1 hour sub-normothermic preservation, we first examined whether or not systemic administration of MSCs immediately after reperfusion improves transplant recipient survival and renal function. Then, we studied impact of a single local administration of MSCs via the renal artery in a CD donor rat renal transplantation (RTx) model. Our finding provides a possible regimen for the treatment of patients with end-stage renal disease that may also rescue other CD organs from damage.

Methods
Animals
Male Lewis (LEW) rats weighing between 260 and 310 g were purchased from Charles River Laboratories Japan (Breeding Laboratories, Kanagawa, Japan). The animals were housed in a temperature- and humidity-controlled environment with a 12-hour light–dark cycle and provided with standard laboratory chow and water ad libitum. Luciferase transgenic (Lac-Tg) and LacZ transgenic (LacZ-Tg) LEW rats, produced by cross-breeding normal LEW rats (Charles River Laboratories Japan, Kanagawa, Japan) and Tg rats, were also used [30]. Rats were housed and bred in the Center for Experimental Medicine at Jichi Medical University and Kitasato University. All experiments were conducted by following established guidelines for animal welfare and were approved by the animal ethics committees of Kitasato University and Jichi Medical University.

Adipose Tissue–Derived MSC Preparation and Culture
To obtain AT-MSCs, adipose tissue isolated from female Luc- or LacZ-Tg rats was minced with scissors and scalpels into pieces smaller than 3 mm. The mixture was separated into two phases by gentle shaking with an equal volume of phosphate buffered saline (PBS) (–). The upper phase (containing stem cells, adipocytes, and blood) was then washed with PBS (–) and enzymatically dissociated with 0.075% collagenase (type I) in PBS (–) for 1 h at 37°C with gentle shaking. The dissociated tissue was mixed with an equal volume of Dulbecco’s Modified Eagle Medium (GIBCO-BRL, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and incubated for 10 minutes at room temperature to separate the solution into 2 phases. The lower phase was centrifuged at 1500 rpm for 5 minutes at 20°C. The cellular pellet was resuspended in 160 mmol/L NaHCl to eliminate erythrocytes and then passed through a 40-mm mesh filter into a new tube. The cells were resuspended in an equal volume of αMEM (GIBCO-BRL, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and incubated for 10 minutes at room temperature to separate the solution into 2 phases. The lower phase was centrifuged at 1500 rpm for 5 minutes at 20°C. The cellular pellet was resuspended in 160 mmol/L NaHCl to eliminate erythrocytes and then passed through a 40-mm mesh filter into a new tube. The cells were resuspended in an equal volume of αMEM (10% FBS) and then centrifuged. When the cells were 70% confluent, they were harvested with 0.05% trypsin-EDTA (Invitrogen, Tokyo, Japan), repeated at 26,104 cells/cm², and cultured for 5 days. MSCs between the fifth and eighth passage were used for the experiments.

CD Donor Kidney Transplantation
Rats were anaesthetized by using isoflurane. In the isogenic RTx model, the left kidney (including the renal artery, vein, and ureter) of the LEW donor rat was isolated and transplanted into the LEW recipient rat. The donor renal artery was anastomosed to the aorta of the recipient, whereas the renal vein was connected to the inferior vena cava by using a side-to-end anastomosis with a continuous running suture. The ureter was anastomosed end-to-end using interrupted sutures. Graft kidneys were transplanted into recipients according to the conditions for the preservation of solutions and timing as described [28]. Anesthesia in recipient rats was induced and maintained throughout surgery using isoflurane, and both kidneys of the recipient rats were nephrectomized at the time of kidney transplantation.

Transplantation of AT-MSCs to CD Donor Kidneys
AT-MSCs in ET-K (Otsuka Pharmaceutical Company, Tokushima, Japan; 2.4 mL) (1.0 × 10⁶ cells/0.5 mL) were injected systematically.
via the penile vein (systemic group; n = 6) after RTx or via the renal artery of the CD donor kidney before angiostomy (local group; n = 8 in each group). AT-MSCs untreated groups were injected ET-K alone.

Assessment of Kidney Function and Survival

Blood samples were obtained for assessment of blood urea nitrogen (BUN) and serum creatinine (CRE) on Day 0 after RTx and then every other day (AU400, Beckman Coulter, Inc., Brea, Calif, United States). The samples were centrifuged for 5 minutes at 3000 rpm and the serum was collected. The survival of recipient rats was checked daily, up to a maximum of 14 days, at which point the rats were sacrificed.

Detection and Quantification of Transgene Expression by Using a Noninvasive In Vivo Imaging System

In vivo luciferase imaging was performed by using the IVIS noninvasive bioimaging system (Xenogen, Alameda, CA, USA) and the IVIS Living Image (Xenogen) software package. Recipient rats were injected with D-luciferin (Promega, Madison, Wis, United States) in the penile vein (30 mg/kg body weight; dissolved and diluted to 15 mg/mL in PBS). Signal intensity was quantified as the photon flux (photons/s/cm²/steradian) in the region of interest [31].

Immunohistochemical Detection of LacZ-Positive Cells

To detect injected LacZ-positive MSCs in transplanted kidneys, some recipient rats were euthanized 24 h after RTx, and their kidneys were harvested. Frozen sections (10 μm) of the kidneys were fixed in 0.2% glutaraldehyde for 10 min at room temperature and incubated in a solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal; Sigma-Aldrich, St. Louis, USA), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 2 mM MgCl₂ in PBS at 37°C for 20 hours.

Histological Analysis

According to available evidence, tissue fibrosis appears to be associated with cellular edema and the cellular edema was produced from ischemia/reperfusion injury [32–34]. Therefore, fibrosis
Score was evaluated. Some rats surviving until day 14 post-RTx were euthanized and their kidneys were harvested, fixed in 10% formalin, and then embedded in paraffin. Two-micron-thick paraffin-embedded sections were stained with azan stain for the assessment of parenchymal fibrosis. In accordance with previous studies [35], the dimensions of the interstitial collagen deposition relative to total interstitium, were analyzed quantitatively in 20 high-power fields of the cortical area per section using BZ-II; Analyzer software (Keyence, Osaka, Japan) on a BIOREVO BZ-9000 microscope (Keyence). The number of pixels with the predetermined color of tone were counted in each section, and then automatically converted into dimensions. We used as a control group (n = 3) that received fresh kidneys.

AT-MSCs were injected systemically via the penile vein (n = 3) after RTx or via the renal artery of the CD donor kidney before angiostomy (n = 3). Some rats surviving (n = 2 in each group) until 3 months post-RTx were histopathologically examined to investigate the presence of teratomas or other abnormalities in the grafts and other organs.

Statistical Analysis

Results are given as mean ± SD. Statistical analyses were conducted by using Student t test for continuous data and the Mann–Whitney test for discontinuous data. Histopathological scores were evaluated by one-way ANOVA. A P value of .05 was considered to be statistically significant.

RESULTS

Survival of CD Kidney Graft Recipients Systemically Treated With MSCs Immediately After Reperfusion

AT-MSCs in ET-K (1.0 × 10⁶ cells) were systemically injected to bilaterally nephrectomized recipient LEW rats that had received a marginal kidney graft (n = 6). There was no direct evidence of pulmonary embolism at this dosage of MSC. Three out of 6 rats survived for more than 7 days after RTx, but there was no beneficial effect on recipient survival (survival in days: 1, 4, 7, 8, >14, >14), as shown previously [28].
In vivo bioimaging showed injected AT-MSCs trapped in the lung immediately after administration, and the number of AT-MSCs fell below measurable limits within 5 days (Fig 1A and B). Our previous study of systemic injection of MSCs in normal rats showed that higher doses of MSCs were often lethal, as others have described [36]. In consideration of the adverse effects associated with systemic injection, the following experiment was conducted.

**Survival of CD Kidney Graft Recipients Locally Treated With MSCs and Assessment of Kidney Function of Surviving Rats**

The impact of local MSC treatment of CD kidney grafts on recipient survival is summarized (Fig 2). There was no significant difference in survival rate at post-operative day (POD) 12, but at POD14, survival in the AT-MSC treatment group (MSC[+] 75%) was improved compared with that in the AT-MSC nontreatment group (MSC[−]; 50%).

BUN (MSC[+]) group; 144.9 ± 5.9, MSC(−) group; 132.3 ± 9.7) and CRE (MSC[+]) group; 2.8 ± 0.1, MSC(−) group; 3.0 ± 0.3) levels in the surviving rats showed the same peak levels for both groups at POD2; however, these markers were significantly reduced (P < .05) in the MSC[+] group (BUN; 89.7 ± 7.6, Cre; 1.55 ± 0.2) compared with the MSC(−) group (BUN; 135.4 ± 18.2, Cre; 2.4 ± 0.3) at POD4. BUN and Cre of all surviving rats recovered to a nearly normal range by day 14.

Luminescence-positive MSCs were detected at very low levels at the graft site immediately after RTx and remained at 24 hours after reperfusion (Fig 3A). LacZ staining showed transplanted AT-MSCs in the proximal tubules of grafts in rats killed at 24 hours after RTx (Fig 3B). However, neither luminescence-positive cells at POD7 nor LacZ-positive cells in the longer-surviving rats were seen (data not shown).

Fibrosis in the kidneys of rats surviving until day 14 was investigated by using azan staining and assigned the dimensions of the interstitial fibrotic deposition relative to total interstitium. The fibrotic area for each group were significantly increased compared with those of a control group (9.5 ± 0.69) that received fresh kidneys (P < .05; Fig 4): MSC[+] group (19.98 ± 3.30), MSC(−) group (26.3 ± 4.31). The marginal graft kidneys of the 1-hour CD group were fibrotic, suggesting severe tissue injury. This difference between MSC[+] group and MSC(−) group was not statistically significant (P > .05), but MSC[+] group had a tendency to decrease the fibrosis compared with MSC(−) group.

In the separated animals, we observed a possible effect of MSC on teratoma formation. No rats had detectable teratoma, infarction, or other abnormalities in the grafts or other organs at 3 months after RTx.

**DISCUSSION**

To expand the marginal donor pool to include CD donors, sub-normothermic and normothermic perfusion have been extensively studied in heart, liver, lung, and kidney in large animal models [37]. In particular, Berendsen et al showed that ischemically damaged livers (warm ischemic time, 1 hour) could be regenerated effectively by using sub-normothermic temperature management [38]. Brasile et al reported that warm perfusion using Belzer’s solution at 32°C supported better oxygen utilization and raised the metabolic rate in dog kidney maintained ex vivo [39]. Furthermore, use of an automatic perfusion system under normothermic conditions appears to restore both the renal function and transplantability of kidneys in both pig and human [40,41].

Clinical trials in Europe for immune disorders such as Crohn’s or acute graft-versus-host disease utilizing auto or allogeneic MSC injection indicate a marked therapeutic effect with the use of MSCs [42-44]. MSC therapy has been extensively applied therapeutically for organ transplantation [12] and there is a potential clinical application of MSCs in marginal donor grafts to avoid the ischemia–reperfusion injury associated with solid organ transplants. However, Eggelhofer et al reported that viable donor MSCs infused via the vein were present in the lungs up to 24 hours after infusion, after which they disappeared [45]. They suggested that MSCs might not survive long term in the recipient animals [45]. Furthermore, the induction of ischemia–reperfusion injury in the liver did not trigger the migration of viable MSCs to the liver [45]. Our results also showed that AT-MSCs infused via the vein were disappeared from lung between 24 hours and day 3 after infusion, which was suggested the possibility that systemically infused AT-MSCs were dead at an early point after infusion and did not migrate into the injured graft kidney.

In addition, intravenous infusion of MSCs in mice triggers a systemic inflammatory response that originates in the lungs and proceeds systemically [46] and lethal pulmonary thromboembolism has been seen in some patients after intravenous AT-MSC administration [25]. Tatsumi et al reported that AT-MSCs triggered thrombus formation around the right ventricle and pulmonary arteries and initiated pulmonary embolism immediately after administration in mice [26]. The mortality rate and adverse events after intravenous-injection of AT-MSCs appear to be enhanced in a cell number–dependent manner. Death from AT-MSC–induced pulmonary embolism occurs within 24 h after injection [26]. Considering these two research areas, we have focused on MSC therapy in CD donor grafts. In separate studies, we assessed intravenous injection therapy using autologous AT-MSCs in rat, cat, and pig. Multiple injection of AT-MSCs has some practical effect on the migration of stem cells to the target organ in rat [47], but it enhanced the risk of lung infarction and death caused by pulmonary embolism and cardiac failure in rats and cats (data not shown). Pig models show elevation of portal vein or pulmonary artery pressure when autologous AT-MSCs are injected through the portal vein or pulmonary artery respectively using a catheter. Therefore, to investigate the restorative effects of AT-MSCs in CD donor kidneys, we locally administered AT-MSCs via the graft’s renal artery.
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ex vivo before angiostomy. Our rat model of CD kidney transplantation showed that a graft kidney left for 1 h after CD was able to function after 1-hour normothermic preservation in ET-K. A warm solution (extracellular-type containing trehalose) may achieve a more rapid clearance of blood from the microcirculation, whereas a warm preservation solution may reduce vasoconstriction and cell membranes stiffening in both endothelial cells and the cellular components of the blood [48]. These actions may ameliorate the no-reflow phenomenon, which is an important mechanism in ischemia–reperfusion injury [48]. For injected MSCs in marginal kidney grafts, a non-cold setting is considered to be beneficial for the secretion of MSC cytokines.

Systemic injection of AT-MSCs can lead to pulmonary embolism; however, in the current study, local injection of AT-MSCs did not cause death by pulmonary embolism and the AT-MSCs remained in the graft kidneys, especially in the proximal tubules, after reperfusion. The proximal tubule is the region most sensitive to ischemia [49]. LacZ staining showed that AT-MSCs migrated to injured tubules in the normothermic-preserved CD kidney and IVIS showed that they remained in the graft kidneys 24 hours after reperfusion. We therefore hypothesize that AT-MSCs might accumulate around injured tubules and produce protective cytokines such as hepatocyte growth factor and fibroblast growth factor [50]. These parked cells gradually reduced in number and disappeared without tumor formation, as previously shown [51].

Regarding the long-term safety and efficacy of MSC therapy, the following reports are important. MSCs injected locally or via vessels are expected to have tissue regenerative, immuno-modulatory, and anti-inflammatory effects. However, 2 South Korean patients, who underwent therapy outside South Korea, were reported to have died of causes related to stem cell therapy [25]. One of the major risks of MSC therapy is pulmonary embolism soon after treatment. It is important to note that MSCs suspended in saline or culture media become sedimentary and begin to aggregate in less than 1 hour, thus increasing the risk of embolism-related complications after intravenous MSC injection. Our preclinical study in pig revealed that intraportal injection of MSCs (1 × 10⁷ cell/kg) in normal saline (200 mL) causes portal emboli followed by death 3 days after administration (data not shown). The injection of MSCs causes the portal pressure to rapidly increase to 24 mm Hg, and the post-mortem patholgy of the liver shows massive emboli in the portal vein. In a recent investigation, we successfully identified a component common in commercialized intravenous solutions that suppresses the aggregation of MSCs. These findings will surely assist in circumventing emboli during the acute phase of MSC therapy; the quality of MSCs and their functions will be scrutinized hereafter.

MSC therapy has been shown to decrease fibrosis in several organs in animal models [52–54]. However, a case of lupus nephritis treated by direct renal injection of autologous stem cells developed angiomylolyproliferative lesions at the site of injection at 6 months after therapy [51]. Thirabanjasak et al suggest that the development of angiomylolyproliferative lesions is a possible complication of stem cell therapy, although the biologic potential of this lesion is unknown [51].

The immune-histopathologic results from our syngeneic transplant model showed that AT-MSCs had a tendency to prevent fibrosis in CD donor kidneys, these cells do contribute to the prompt recovery of kidney function. MSC therapy may prevent long-term fibrotic changes in the allogeneic transplantation clinical setting [54]. Our long-term observations show that local injection of AT-MSCs does not induce adverse events such as teratoma or infarction 3 months after RTx; therefore, local injection of AT-MSCs is a potentially safer route of administration than high-dosage, intravenous injection.

REFERENCES