Experimental model and design

Lewis rats (Japan SLC) weighing 230 to 250 g were used throughout the experiments, and randomly allocated into two groups: 1) control phosphate-buffered saline (PBS) injection alone (PBS group) and 2) FM-MSCs injection (FM-MSC group).Rats were then subjected to I/R injury. All rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg; Abbott Laboratories, North Chicago, IL), and were subjected to 60 min of renal occlusion using artery clips to clamp the renal pedicles, and were performed right nephrectomy. Occlusion was visually confirmed by a change in kidney color to a paler shade. Reperfusion was initiated with the removal of artery clips and was visually confirmed by noting the subsequent blush. Prior to the removal of the artery clips, rats received PBS or 5×10^5 FM-MSCs obtained from MHC mismatched ACI rats via the tail vein. Rats were sacrificed 6 hrs (n=6 in each group), 12 hrs (n=6 in each group), 24 hrs (n=9 in each group) and 72 hrs (n=8 in each group) after reperfusion. Sham operated rats (sham group; n=6 in each period) underwent the same procedure except that clamping was not done, and received a PBS injection. Blood was also collected at the time of sacrifice, and BUN and serum creatinine levels were measured by SRL (Tokyo, Japan).

To assess the distribution of the injected cells, intravenous transplantation of the same number of FM-MSCs (5×10^5cells) derived from GFP-transgenic Lewis rats were performed in I/R injured ACI rats. 24 hrs after the injection, the rats were sacrificed, and sections of tissues were obtained from the kidney, lung, spleen, and liver, and embedded in paraffin (n=4 for each tissue).

To explore the role of IL-10 in this model, Lewis rats were induced I/R injury and received FM-MSCs transplantation and anti-IL-10 neutralization antibody (0.05μg/g body weight, which is more sufficient dose to neutralize serum IL-10 levels; R&D Systems) at the time of reperfusions. The concentration of IL-10 in serum was determined using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The absorbance was measured by a microplate reader (Bio-Rad, Hercules, CA) at 450 nm.

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Morphology and immunohistochemical staining

Kidney tissues were fixed with 4% phosphate-buffered formalin solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin block. Tissue sections (2μm) were used for periodic acid-Schiff (PAS), or for immunohistochemical staining, which was performed with mouse anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4; Dako, Glostrup, Denmark), mouse anti-CD68 antibody (clone ED-1, Serotec, Oxford, UK), mouse anti-CD3 antibody (BD Biosciences Pharmingen, San Jose, CA), and rabbit anti-GFP antibody (Invitrogen). Following antigen retrieval, endogenous peroxidase activity was quenched with 1.5% H_2O_2 for 10 min. The first antibodies were incubated for 1 hour at room temperature, followed by incubation with DakoCytomation LASB2 System-HRP (Dako). The sections were visualized with 3,3′diaminobenzidine tetrahydrochloride (Dako) and counterstained with hematoxylin. All histological slides were photographed using a digital microscope (BIOREVO BZ-9000;

Keyence, Osaka, Japan). PAS-stained sections were scored by calculation of percentage of tubules in corticomedullary junction that displayed cell necrosis, loss of brush border, cast formation, and tubular dilation as follows: 0, none; 1, <10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, >76%. At least 20 randomly selected fields per rat were scored. The number of ED-1 and CD 3 -positive cells was evaluated by counting stained cells per areas in at least 20 randomly selected areas. The area of interstitial renal fibrosis in the outer medulla was calculated as a percentage using a computer-aided manipulator (Win Roof; Mitani, Fukui, Japan).The α-SMA staining percentage of total area was determined and the mean value of 20 randomly selected interstitium was calculated. To evaluate the distribution of GFP-positive administered cells, we counted all the GFP-positive cells in one randomly selected section from each organ and an overall average for all rats was calculated.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining.

TUNEL staining was performed using the in situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan), according to the manufacturer's instructions. Briefly, sections were deparaffinized, subjected to antigen retrieval, and were then incubated with 1.5% H_2O_2 for 10 min, followed by incubation with TdT enzyme solution for 90 min at 37°C. The reaction was terminated by incubation in a stop/wash buffer for 30 min at 37°C. The number of TUNEL-positive cell nuclei and the total numbers of cell nuclei stained with hematoxylin were counted in 20

random areas, and the percentage of TUNEL-positive nuclei against total cell nuclei was then calculated.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from the kidney cortex using an RNeasy mini kit (Qiagen, Hilden, Germany). Obtained RNA was reverse-transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen). PCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). ß-actin transcript used as an internal control . Primers were as follows: Monocyte Chemotactic Protein-1 (MCP-1); 5′-atgcagttaatgccccactc-3′ (forward), 5′-ttccttattggggtcagcac-3′ (back), IL-6; 5′-ccggagaggagacttcacag-3′ (forward), 5′-cagaattgccattgcacaac -3′ (back), Transforming Growth Factor-ß (TGF-ß); 5′- ctactgcttcagctccacagaga -3′ (forward), 5′- accttgggcttgcgacc -3′ (back), Type I collagen; 5′- aatggtgctcctggtattgc -3′ (forward), 5′- ggttcaccactgttgccttt -3′ (back), ß-actin; 5′- gccctagacttcgagc -3′ (forward), 5′- ctttacggatgtcaacgt -3′ (back).

Statistical analysis

All data were expressed as mean ± SEM. Comparisons of parameters among the three groups were made by one-way analysis of variance (ANOVA), followed by Tukey's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Protection of renal function by FM-MSCs transplantation

We examined the effect of FM-MSCs on renal function (Fig.1). Ischemia-reperfusion (I/R) injury induced significantly elevated serum creatinine level (1.51±0.2 mg/dl; PBS group) 24 hrs later compared to the sham-operation (0.31±0.02 mg/dl). FM-MSCs treatment significantly reduced this increase (0.77±0.05 mg/dl, $p<0.05$ vs. PBS group; Fig. 1A). In addition, we observed the increase of BUN at 24 hrs after I/R injury (92±10.3 mg/dl) in PBS group, while FM-MSCs administration significantly inhibited (56.7±7.8 mg/dl, $p<0.05$ vs. PBS group; Fig. 1B). Similarly, FM-MSCs transplantation also improved the renal function at 72 hrs after I/R injury (creatinine; 0.95± 0.13 mg/dl in PBS group vs. 0.53± 0.06 mg/dl in FM-MSC group, $p<0.05$, BUN; 93.2± 13.9 mg/dl in PBS group vs. 48.1± 5.4 mg/dl in FM-MSC group, $p<0.05$).

Effects on histological changes and apoptosis of tubular epithelial cells

We then tested the effect of FM-MSCs on histological changes (Fig.2). PAS staining of the kidney sections obtained from PBS-treated I/R-injured rats at 24 hrs exhibited marked disruption of normal tubular morphology, including widespread degeneration of tubular architecture, tubular dilatation, swelling, and luminal congestion with loss of the brush border (PAS score; 2.53± 0.16 in PBS group vs. 0.07± 0.14 in sham group, $p<0.001$; Fig. 2A, B and G). In contrast,

FM-MSCs treatment reduced severity with regard to the characteristic histological changes of I/R injury, including tubular atrophy (1.31± 0.06, $p<0.001$ vs. PBS group; Fig.2C and G). At 72 hrs after reperfusion, PBS-treated rats exhibited more severe tubular injury than FM-MSCs transplanted rats (3.52± 0.15 in PBS group vs. 1.71± 0.08 in FM-MSC group, $p<0.001$ Fig. 2G). Moreover, TUNEL-positive apoptotic cells were significantly increased in PBS-treated rats at 12 and 24 hrs after I/R injury (12hrs; 12.78± 0.43%, 24hrs; 11.34± 0.59%, $p<0.001$ vs. sham group) compared with sham-operated rats (12 hrs; 0.16±0.02%, 24 hrs; 0.08± 0.01%), while FM-MSCs treatment significantly reduced the apoptotic cells (12 hrs; 4.98± 0.56%, 24 hrs; 2.14± 0.20%, $p<0.01$ vs. PBS group) (Fig.2D-F,H). Similarly, I/R induced apoptosis (1.08± 0.10% in PBS group vs. 0.10± 0.02% in sham group, $p<0.01$) was significantly attenuated by FM-MSCs transplantation (0.74.± 0.07%, $p<0.01$) at 72 hrs after reperfusion.

Inhibition of T cells and macrophages infiltration by FM-MSCs transplantation

Next, we examined the effect of FM-MSCs on T cells and macrophages infiltration into I/R-injured kidney (Fig.3). At 6 hrs after reperfusion, infiltrating CD 3-positive T cells were significantly increased in PBS-treated kidneys (8.31± 0.41 cells/ field, $p<0.001$ vs. sham group; Fig.3B) compared with sham-operated kidneys (3.1± 0.2 cells/ field; Fig.3A). On the contrary, FM-MSCs treatment significantly suppressed the T cells infiltration (5.8± 0.3 cells/ field, $p<0.001$ vs. PBS group; Fig. 3C,G). A similar result was also observed at 12 hrs after reperfusion (PBS group 4.14± 0.30 cells/ field vs. FM-MSC group 2.98± 0.27 cells/

field, $p<0.001$).

We then investigated the effect of FM-MSCs on interstitial macrophages infiltration, which is associated with interstitial damage and fibrosis.ED-1-positive macrophages were increased in the PBS group at 12, 24 and72 hrs after reperfusion (134.6± 3.8, 387.9± 17.0 and 812.7± 50.8 cells/ field, respectively, $p<0.001$ vs. sham group) compared to sham-operated rats (11.4± 1.1, 7.2± 0.7 and 20.8± 2.2 cells/ field, respectively), while FM-MSCs transplantation significantly suppressed interstitial ED-1-positive macrophages infiltration (105.6± 3.0, 310.6± 14.4 and 438.5± 33.2 cells/ field, respectively, $p<0.001$ vs. PBS group) (Fig3D-F,H). As we observed the inhibition of T cells and macrophages infiltration, we examined various pro-inflammatory cytokines and chemokines expression in I/R-injured kidneys. qRT-PCR analysis showed that I/R-injury induce increased MCP-1 expression at 6, 12 and 24 hrs after reperfusion (4.36± 0.97-, 3.35± 0.73- and 7.62± 0.97-fold, respectively, $p<0.05$ vs. sham group), and FM-MSCs transplantation significantly suppressed this increased expression (2.02± 0.13-, 1.56± 0.35- and 4.40± 0.54-fold, respectively, $p<0.05$ vs. PBS group; Fig.3I). FM-MSCs administration also significantly suppressed IL-6 mRNA expression at 6, 12 and 24 hrs after reperfusion (9.10± 1.43-, 4.69± 0.92- and 1.82± 0.25-fold in FM-MSC group, respectively, $p<0.05$ vs. 16.22± 2.45-, 7.61± 1.21- and 3.34± 0.39-fold, respectively in PBS group; Fig. 3J).

Effects on interstitial phenotypic changes in kidney after I/R injury

We next studied the effect of FM-MSCs on interstitial phenotypic

alteration (Fig.4), assessed by immunohistochemical staining using anti-α-SMA antibody, which are associated with interstitial damage and fibrosis. α-SMA expression was limited to vessels at 24hrs after I/R injury in sham-operated(0.04± 0.01%), PBS-treated (0.06± 0.01%) and FM-MSCs-treated kidney (0.06± 0.01%). However, interstitial α-SMA expression significantly increased in PBS-treated kidney at 72 hrs (10.09± 0.41%, $p<0.001$ vs. sham group) compared to the sham-operated kidney (0.56± 0.04%), while FM-MSCs transplantation suppressed the interstitial α-SMA expression (4.44± 0.38%, $p<0.001$ vs. PBS group; Fig. 4A-D). Similarly, qRT-PCR analysis revealed that FM-MSCs transplantation significantly decreased TGF-β and type I collagen mRNA expression, which are related with renal interstitial fibrosis, at 72 hrs after reperfusion (1.19± 0.14-fold and 1.61± 0.47-fold, respectively, $p<0.05$ vs. PBS group) compared with the PBS group (2.32± 0.44-fold and 6.28± 1.87-fold, respectively, $p<0.05$ vs. sham group; Fig. 4E,F).

Engraftment of intravenously injected FM-MSCs in rats with I/R injury

We previously reported that intravenously injected FM-MSCs were rarely observed in kidney at 7 days after transplantation. To investigate the distribution of intravenously administered FM-MSCs at the early period, we intravenously administrated FM-MSCs, which derived from GFP transgenic Lewis rats, into I/R-injured ACI rats at the time of reperfusions (Fig.5). GFP-positive FM-MSCs were faintly detected in the kidney sections at 6hr (28.1± 1.1 cells/cm^2) and 24 hr (11.9± 1.4 cells/cm^2; Fig. 5A) as well as liver (at 6hr; 32.1± 1.4 cells/cm^2, at 24hr;

41.1± 3.2cells/cm², Fig. 5B) and spleen (at 6hr; 26.2 ± 1.8 cells/cm², at 24hr; 27.6± 3.3 cells/cm², Fig. 5C). On the contrary, we observed more GFP-positive FM-MSCs in lung (at 6hr; 335.9± 18.1 cells/cm², at 24hr; 124.1± 8.5 cells/cm², Fig. 5D).

Effects of IL-10 neutralizing antibody in FM-MSCs transplanted rats with I/R injury

As we observed rare FM-MSCs in kidney, we then examined the FM-MSCs-derived humoral therapeutic factor (Fig.6). In our previous study, we confirmed that FM-MSCs secreted a significant amount of PGE2 in FM-MSCs supernatant (888.1± 123.3pg/ml) (43). As PGE2 enhanced the secretion of IL-10 from immune cell, we measured serum IL-10 levels, and found significant increase of IL-10 in the FM-MSC group at 24 hrs after reperfusion (113.0±13.2pg/ml), while serum IL-10 was not detected in PBS-treated group. Therefore, we confirmed if IL-10 is a key mediator in this model. FM-MSCs-treated rats, received anti-IL-10 neutralization antibody, exhibited undetected serum IL-10 (<10pg/ml) levels, and the renoprotective effect of FM-MSCs on I/R injury was cancelled (creatinine; 1.24±0.2 mg/dl, p=0.32, vs. PBS group, BUN; 83.3± 9.1 mg/dl, p=0.54, vs.PBS group; Fig. 6A,B).

DISCUSSION

In this study, we investigated the renoprotective effects and the mechanism of FM-MSCs in AKI model; 1) intravenous injection of allogenic FM-MSCs improved renal function in rats with I/R injury, 2) allogenic FM-MSCs administration inhibited tubular apoptosis, interstitial fibrosis, and infiltration of macrophages and T cells in a rat model of I/R injury, 3) administered FM-MSCs mainly homed into lung, but not into kidney, 4) serum IL-10 levels were increased in FM-MSCs-treated rats, but 5) renoprotective effects of FM-MSCs are abolished by anti-IL-10 neutralization antibody

In this study, we employed FM-MSCs as a new type of MSCs. Several studies have shown beneficial effects of BM-MSCs transplantation in AKI model rats (5,19,20,25-28,41). However, clinical application of autologous BM-MSCs is limited because of several problems such as their invasiveness, inadequate cell numbers, and donor-site morbidity (45). On the other hand, FM tissue possesses great advantages due to its abundance and easy accessibility, and has been shown to be rich sources of MSCs (21,35). In our previous reports, we demonstrated that allogeneic FM-MSCs as well as autologous BM-MSCs are suitable cell sources for tissue regeneration using a rat hind-limb ischemia model. Therefore, we propose that allogeneic FM-MSCs might be a good alternative to autologous BM-MSCs.

Here, we suggested that one of the therapeutic mechanisms of FM-MSCs is due to anti-inflammatory effect via systemic IL-10 production. The underlying immunomodulatory mechanisms of MSCs remains largely unknown whether direct cell-cell contact or humoral factors are involved. Previously, we and others

reported that PGE2 is one of the key modulators for the MSCs-induced anti-inflammatory response (7,43). Nemeth et al. reported that BM-MSCs reprogrammed macrophages by releasing PGE2, and thereby IL-10, secreted from reprogrammed macrophages, attenuated septic conditions. In this study, we showed that FM-MSCs-transplanted rats exhibited increased IL-10 in I/R injured rats at 24 hrs at reperfusion. On the contrary, injecting anti-IL-10 neutralization antibody cancelled the renoprotective effect of FM-MSCs on I/R injury. These results indicated that FM-MSCs ameliorated renal I/R injury via IL-10 secretion. IL-10 is a pleiotropic cytokine with many immunosuppressive effects, and this potent anti-inflammatory cytokine could suppress the activation of neutrophils and monocytes, and thereby prevent their production of chemokines and cytokines in animals and humans (12,14,31,32). However, the fact that IL-10 neutralizing antibody did not perfectly cancel the renoprotective effects suggests that IL-10 is not solely responsible for FM-MSCs-derived therapeutic effects.

We demonstrated that FM-MSCs transplantation inhibited macrophages infiltration in rat I/R injured kidneys, compared with PBS-treatment. Previous studies have focused on anti-inflammatory effect of MSCs, which are able to inhibit systemic and local inflammatory responses in several models of inflammatory conditions (3,18,28). Inflammation also plays an important role in I/R injured kidney, and macrophages are key participants in I/R injury (15). Monocytes/macrophages infiltration, and monocyte/macrophage chemoattractants, such as MCP-1 (33,40,47) as well as macrophage-associated cytokines, such as IL-6,and TNF-α (40), were related in the I/R injured kidney. In addition, inhibition of CCR2, receptor for MCP-1, markedly ameliorated disease

manifestations in I/R injured mice (17). In this rat I/R injury model, we demonstrated that administered FM-MSCs suppressed MCP-1 and IL-6 expression. Previously, we reported that allogeneic FM-MSCs administration significantly attenuates macrophages infiltration in rats model of acute glomerulonephritis (43). Therefore, FM-MSCs could modulate local inflammatory responses by inhibiting MCP-1 and IL-6 expression, and contribute to the preservation of renal function and structure in renal I/R injury model.

In this study, T cells infiltration occurred at 6 hrs and vanished by 24 hrs, and we demonstrated that FM-MSCs significantly reduced T cells infiltration. In addition to potent anti-inflammatory effect, many reports have highlighted the potential immunomodulatory effect of MSCs (1,30), such as suppression of T cells activation and proliferation both in vitro and in vivo (4,23). Recent studies have also identified T cells as important mediators (8,36,37,46), especially so called "hit-and-run" attack, early T cells infiltration at 4-6 hrs and resolution by 24 hrs (2,15,24). Our observation was consistent with this phenomenon. Therefore, we suggest that renoprotective effect of FM-MSCs, in part, may be due to the inhibition of systemic inflammatory responses, by controling early T cells infiltration after I/R by attenuating the T cells activation during the early reperfusion period.

In conclusion, this study showed that FM-MSCs transplantation ameliorated renal function in a rat model of AKI. Because FM-MSCs is available non-invasively in large amounts, we suggest that allogeneic FM-MSCs could provide a new therapeutic strategy for the treatment of AKI.

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Figure legends

Fig.1. Efects of FM-MSCs transplantation on renal function

Effect of FM-MSCs transplantation on renal function was summarized. Creatinine (A) and BUN (B) were examined 6, 12, 24, and 72hrs after sham operation (sham) or ischemia-reperfusion injury treated with PBS (PBS) or FM-MSCs transplantation(FM-MSC). *p<0.05 vs. sham group, †;p<0.05 vs. PBS group.

Fig.2. Efects of FM-MSCs transplantation on renal injury

Representative micrographs of PAS and TUNEL staining in sham (A, D), PBS (B, E) and FM-MSC (C, F) groups at 24 hrs after reperfusion. Quantitative analysis of tubular damage (G) and the number of TUNEL-positive cells (H) were summarized. All stained sections were analyzed in twenty non-overlapping low-power fields at ×200 magnification. Scale bars=50μm. ** p<0.01, ***p<0.001 vs. sham group; †† p<0.01, ††† p<0.001 vs. PBS group.

Fig.3. **Effects of FM-MSCs transplantation on T cells and macrophages infiltration**

Representative images of CD 3 staining at 6hrs and ED-1 staining at 72hrs from kidney sections of sham (A, D), PBS (B, E) and FM-MSC (C, F) groups were shown. Quantification of CD 3-positive cells (G) and ED-1-positive cells (H) was analyzed. Original magnification, ×200. Scale bars=50μm Arrows indicate the

positive staining for CD 3.

Real-time PCR showed MCP-1 (I) and IL-6 (J)mRNA level at 6,12 and 24 hrs. Result was expressed as relative expression against the expression in sham-operated rats. *; $p<0.05$, ***; $p<0.001$ vs. sham group, † ;$p<0.05$, ††† ;$p<0.001$ vs. PBS group.

Fig.4. **Effects of FM-MSCs transplantation on phenotypic alteration**

Representative images of α-SMA staining were shown in kidney sections of sham (A), PBS (B) and FM-MSC (C) groups at 24hrs and 72hrs. Quantification of α-SMA-positive area (D) was analyzed. Original magnification, ×200. Scale bars=50μm. Real-time PCR showed TGF-β mRNA level (E), and Type I collagen at 72hrs (F). Result was expressed as relative expression against the expression in sham-operated rats. *; $p<0.05$, ***; $p<0.001$ vs. sham group, † ;$p<0.05$, ††† ;$p<0.001$ vs. PBS group.

Fig.5. **Distribution of intravenously transplanted FM-MSCs in I/R injured rats**

Representative micrographs showing localization of administrated GFP-positive FM-MSCs in kidney (A), liver (B), spleen (C) and lung (D) in a rat with I/R injury. Arrows indicate the positive staining for GFP. Original magnification, ×400. Scale bars=50μm

Fig.6. **Effects of anti-IL-10 neutralizing antibody on FM-MSCs transplantaion treatment**

Injection of anti-IL-10 neutralization antibody cancelled the effectiveness of FM-

MSC in creatinine(A) and BUN(B). *; $p<0.05$, **; $p<0.01$ vs. sham group, † ;$p<0.05$ vs. PBS group.

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