Human Peripheral Blood Mononuclear Cells Incubated in Vasculogenic Conditioning Medium Dramatically Improve Ischemia/Reperfusion Acute Kidney Injury in Mice

Takayasu Ohtake1,2, Shuzo Kobayashi1,2, Shimon Slavin3, Yasuhiro Mochida1, Kunihiro Ishioka1, Hidekazu Moriya1, Sumi Hidaka1, Ryo Matsuura4, Maki Sumida4, Daisuke Katagiri4, Eisei Noiri4, Kayoko Okada5, Hiroshi Mizuno5, and Rica Tanaka5

Abstract

Acute kidney injury (AKI) is a major clinical problem that still has no established treatment. We investigated the efficacy of cultured human peripheral blood mononuclear cells (PBMNCs) for AKI. Ischemia/reperfusion injury (IRI) was used to induce AKI in male nonobese diabetic (NOD/severe combined immunodeficiency) mice aged 7 to 8 wk. PBMNCs were isolated from healthy volunteers and were subjected to quality and quantity controlled (QQc) culture for 7 d in medium containing stem cell factor, thrombopoietin, Flt-3 ligand, vascular endothelial growth factor, and interleukin 6. IRI-induced mice were divided into 3 groups and administered (1) 1 × 10^6 PBMNCs after QQc culture (QQc PBMNCs group), (2) 1 × 10^6 PBMNCs without QQc culture (non-QQc PBMNCs group), or (3) vehicle without PBMNCs (IRI control group). PBMNCs were injected via the tail vein 24 h after induction of IRI, followed by assessment of renal function, histological changes, and homing of injected cells. Blood urea nitrogen and serum creatinine (Cr) 72 h after induction of IRI in the QQc PBMNCs group dramatically improved compared with those in the IRI control and the non-QQc PBMNCs groups, accompanied by the improvement of tubular damages. Interstitial fibrosis 14 d after induction of IRI was also significantly improved in the QQc PBMNCs group compared with the other groups. The renoprotective effect noted in the QQc PBMNCs group was accompanied by reduction of peritubular capillary loss. The change of PBMNCs' population (increase of CD34^+ cells, CD133^+ cells, and CD206^+ cells) and increased endothelial progenitor cell colony-forming potential by QQc culture might be one of the beneficial mechanisms for restoring AKI. In conclusion, an injection of human QQc PBMNCs 24 h after induction of IRI dramatically improved AKI in mice.

Keywords

acute kidney injury, ischemia/reperfusion, CD34, mononuclear cell, QQc culture
Introduction

The high prevalence of acute kidney injury (AKI) and its impact on the prognosis of critically ill patients in the intensive care unit (ICU) is one of the major problems in the field of critical care nephrology. AKI has been reported in 6% to 36% of all ICU patients, and blood purification therapy is required for 0.4% to 3.3% of ICU patients\textsuperscript{1,2}. Regarding the prognosis of ICU patients with severe AKI, a multinational, multicenter study revealed a surprisingly high mortality rate (≥50%) of these patients in many countries all over the world\textsuperscript{521}. However, there is currently no established treatment that promotes kidney repair in patients with severe AKI. Therefore, an effective therapy for AKI is urgently needed.

Cell-based regenerative therapy has been studied in animal models of AKI and there have been some reports of beneficial effects. The cells investigated so far include granulocyte colony-stimulating factor-mobilized peripheral blood CD34\textsuperscript{+} cells\textsuperscript{3} and mesenchymal stem cells (MSCs) derived from bone marrow\textsuperscript{4–8}, adipose tissue\textsuperscript{9–12}, umbilical cord blood\textsuperscript{13,14}, or amniotic fluid\textsuperscript{15}. In addition, progenitor cells generated from human-induced pluripotent stem (iPS) cells have been found to ameliorate AKI induced by ischemia/reperfusion injury (IRI) in mice\textsuperscript{16}.

These cell therapies have been demonstrated to improve the time course of kidney function in animals after AKI. However, no clinical trials have successfully improved AKI with regenerative therapy in humans. When translational research is performed to apply such new clinical treatments, easy accessibility of the cell source, ease of preparing the cells, and cost should be considered. Autotransplantation avoids allosensitization and thus is the safest method of regenerative therapy at present.

Endothelial progenitor cells (EPCs) promote angiogenesis and can be easily collected from the peripheral blood. In the kidneys, blood supply to the nephrons and maintenance of kidney function are regulated by the peritubular capillary (PTC) network; and collapse of this network is thought to be important in the pathophysiology of IRI.

Short-term quality and quantity controlled (QQc) culture of peripheral blood mononuclear cells (PBMCs) is a recently established method for enhancing the number and proliferative capacity of CD34\textsuperscript{+} EPCs\textsuperscript{17,18}. In this study, we examined the effect of human PBMCs incubated in QQc culture medium on AKI in mice by evaluating whether QQc-cultured PBMCs could restore kidney function and reverse tubular/PTC damage in a mouse model of AKI. In most previous IRI studies in animals, cells were injected immediately or shortly after the induction of IRI\textsuperscript{14,10,16}. Delayed cell administration studies are limited\textsuperscript{3}. We conducted cell therapy 24 h after the release of clamped renal pedicles, at which time AKI was thoroughly induced, thereby providing useful implications for future clinical trials.

Materials and Methods

Animals

Male immune-deficient nonobese diabetic (NOD/severe combined immunodeficiency [SCID]) mice aged 7 to 8 wk and weighing 20 to 25 g (Jackson Lab, Kawasaki, Japan) were used for all experiments. The NOD mouse is the model for type 1 diabetes mellitus; however, onset of diabetes in male NOD mice occurs around 150 d after birth, and no mice used in this experiment were diabetic during the experimental period. NOD/SCID mice lack functional T and B cells and are immunodeficient; therefore, rejection of human cells could be neglected, and more pure insight into the protective potential of human cells could be obtained without immunological modulation. Animal care and treatment conformed to institutional guidelines and international laws and politics. The experimental protocol was approved by the Center for Animal Research at Juntendo University.

Human PBMCs

Healthy male volunteers aged 20 to 30 y provided peripheral blood (70 mL) after giving oral and written informed consent. The procedure for obtaining informed consent was approved by the ethics committee of Juntendo University (2013086) and Shonan Kamakura General Hospital (TGE00352-024).

Serum-free QQc Culture

Human PBMCs were isolated as described previously, and \(1 \times 10^5\) PBMCs were added to each well of a 24-well plate (BD Falcon, Bedford, MA, USA) and cultured in serum-free QQc culture medium for 7 d\textsuperscript{17–19}. Briefly, QQc culture medium is an optimized combination of growth factors and cytokines (20 ng/mL thrombopoietin, 20 ng/mL interleukin (IL) 6, 100 ng/mL stem cell factor (SCF), 100 ng/mL Flt-3 ligand, and 50 ng/mL vascular endothelial growth factor; all from Peprotech, Rocky Hills, NJ, USA) in serum-free stem cell medium (Stemcell Technologies, Vancouver, Canada). QQc culture was performed in this medium for 7 d, which has been shown to dramatically and optimally expand and enhance the vasculogenic potential of EPCs.

EPC Colony-forming Assay and Cell Population Assay

The vasculogenic potential of human PBMCs after QQc culture was assessed by the EPC colony-forming assay as described previously\textsuperscript{17–19}. This colony-forming assay was designed to separate total colony-forming units (CFUs) into 2 different types of EPC-CFUs, which were primitive (small cells) and definitive (large cells). The definitive EPC-CFUs (dEPC-CFUs) are a predominantly vasculogenic cell population with greater differentiation potential. Briefly, \(2 \times 10^5\) human PBMCs were seeded into a 35-mm hydrophilic tissue culture dish. After 14 d, total EPC-CFUs, primitive EPC-CFUs, and dEPC-CFUs were counted in a blinded manner by 2 investigators. The experiments were performed in triplicate.
In the cell population assay, freshly isolated non-QQc PBMNCs and QQc PBMNCs were subjected to flow cytometry (FCM) to detect surface antigen positivity of hematopoietic stem or lineage-committed cells as well as endothelial lineage cells as previously reported. The scatter diagram of each non-QQc PBMNC and QQc PBMNC population was gated into 3 cell size populations of lymphocytes, monocytes, and larger cells. The percent positivity of a hematopoietic cell population for each gate in non-QQc PBMNCs and QQc PBMNCs was evaluated and then calculated relative to that of the total cells in the 3 gates. The ratio of the percent positivity of the total cells of QQc PBMNCs to that of the total cells of non-QQc PBMNCs was further calculated for each cell population. FCM analysis was performed using the LSR-Forresta cell analyzer (BD Biosciences, San Jose, CA, USA) and FlowJo software, version 7.6.5 (Tomy Digital Biology Co. Ltd., Tokyo, Japan). Antibodies recognizing the cell populations were used as described previously.

**IRI Model and Cell Therapy**

Mice were kept under a 12-h light–dark cycle at a temperature of 25 °C and received water and food ad libitum. IRI was induced as reported previously. Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital and buprenorphine hydrochloride and placed on a heating pad (37 °C) for 30 min. The kidneys of the anesthetized mice were exposed through flank incisions, and nontraumatic clamps were placed across the bilateral renal pedicles. After confirming a dusky color of both kidneys, the kidneys were replaced in the retroperitoneum for 30 min. The clamps were then removed and reperfusion of the kidneys was confirmed. Blood samples were withdrawn from the tail vein at baseline, 24 h, 48 h, 72 h, and 7 d after IRI, and serum was kept at −80 °C until analysis. Blood urea nitrogen (BUN) was measured using a BUN Test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and serum Cr was determined by HPLC as described previously.

**Evaluation of Kidney Function**

To assess the changes in kidney function, serial blood samples were withdrawn from the tail vein at baseline, 24 h, 48 h, 72 h, and 7 d after IRI, and serum was kept at −80 °C until analysis. Blood urea nitrogen (BUN) was measured using a BUN Test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and serum Cr was determined by HPLC as described previously.

**Kidney Tissue Preparation and Evaluation**

Kidney tissues were obtained from mice anesthetized by intraperitoneal injection of pentobarbital and buprenorphine hydrochloride. For light microscopy, tissues were fixed in 10% neutral-buffered formalin, transferred to 70% ethanol, and then processed to yield paraffin sections (2 μm thick). Sections were stained with hematoxylin and eosin for analysis of tubular damage and with Masson’s trichrome stain for calculation of the interstitial fibrosis area. Tubular damage, including epithelial necrosis, tubular dilatation, cast formation, and loss of the brush border, was evaluated as described previously. Ten fields per mouse incorporating the cortex and outer medulla were captured by digital imaging (×200), and a semiquantitative evaluation was performed by scoring for the damaged area in each field as follows: 0: 0%; 1: <10%; 2: 10% to 25%; 3: 25% to 50%; 4: 50% to 75%; and 5: >75%. Scores were assessed on 3 mice in each group at each time point. Interstitial fibrosis was evaluated quantitatively with imaging software (cellSens®, Olympus, Japan). Fourteen days after IRI, Masson’s trichrome-stained kidney sections were analyzed from 3 mice in the QQc PBMNCs, non-QQc PBMNCs, and vehicle control groups. At ×200 magnification, the area of blue Masson’s trichrome staining (fibrosis) was automatically captured and calculated by the software. The interstitial fibrosis area was expressed as the blue area/total tissue area in 10 fields per kidney in each mouse.

**Homing of Injected Human Cells to the Kidney and Other Organs**

To test the homing of injected human PBMNCs to the kidney, lung, spleen, and bone marrow, QQc or non-QQc PBMNCs were labeled with the cell tracker PKH67 before injection (Green Fluorescent Cell Linker Kit, Sigma-Aldrich, IL, USA). Cells were incubated with fluorescent PKH for 5 min at 25 °C, washed 3 times, and suspended in 0.1 mL of saline before injection. The kidneys, lungs, and spleens harvested from the anesthetized mice were fixed overnight in 4% paraformaldehyde in a darkroom at 4 °C. Fixed tissues were processed in a graded sucrose series and preserved in optimum cutting temperature (−80 °C) until analysis. Four-μm-thick cryosections were then cut, and the number of PKH-positive cells per long axis slice was counted in each organ of 3 mice in each group (4 fields per mouse) at each time point. To count human PKH-positive cells in bone marrow, femora harvested from the mice were immersed in 4% paraformaldehyde in a darkroom at 4 °C and cryosections were prepared for analysis using the Kawamoto method.

**PTC Loss**

In 4-μm-thick cryosections of the kidneys, vessels were labeled by using a purified anti-mouse CD31 antibody (1:100, BD
Pharmingen, San Diego, CA, USA) that did not cross-react with human antigens. Fifteen to 20 fields incorporating the cortex and outer medulla were captured by digital imaging (C2 400). Each image was then divided into 252 squares using a grid. Each square without a PTC (capillary loss) was scored and the final score was represented as a percentage PTC loss.

Expression of mouse vascular endothelial antigen in human PBMNCs was evaluated using an anti-mouse CD31 antibody that did not cross-react with human antigens (BD Pharmingen). Four-micron-thick cryosections of kidneys from the QQc PBMNCs group taken 24 h and 14 d after induction of IRI were stained with anti-mouse CD31 antibody.

Statistical Analysis
All data are expressed as mean ± standard error. Comparison between 2 groups was made by Mann–Whitney U test, and comparison among 3 groups was made by analysis of variance followed by post hoc test. SPSS statistics version 11.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis on a personal computer, and P values < 0.05 was considered significant.

Results
QQc PBMNCs Dramatically Restored Kidney Function
Changes in kidney function are shown in Fig. 1. Twenty-four hours after induction of IRI, the BUN levels did not differ among the IRI control (n = 13), non-QQc PBMNCs (n = 13), and QQc PBMNCs groups (n = 13). However, the QQc PBMNCs group showed dramatic improvement of BUN 48 h after injection of 1 × 10^6 cells compared with that in the IRI control group (99.5 ± 39.4 mg/dL in the IRI control group vs. 36.1 ± 4.3 mg/dL in the QQc PBMNCs group, P < 0.05; Fig. 1A). Serum Cr also showed significant improvement 48 h after cell injection in the QQc PBMNCs group compared with that in the IRI control group (0.89 ± 0.19 vs. 0.25 ± 0.06 mg/dL, respectively, P < 0.05; Fig. 1B). In contrast, non-QQc PBMNCs did not have any beneficial effect on BUN or Cr (Fig. 1A and 1B).

Effect of Cell Therapy on Kidney Damage
Tubular damage was evaluated semiquantitatively by the assessment of epithelial necrosis, tubular dilatation, cast formation, and loss of the brush border. As shown in Fig. 2, all of these tubular damage parameters were significantly improved in the QQc PBMNCs group compared with those in the IRI control group. In contrast, some parameters (cast formation and loss of the brush border) were worse in the non-QQc PBMNCs group compared with those in the IRI control group at 48 and/or 72 h after induction of IRI.

QQc PBMNCs Improve Interstitial Fibrosis in the Recovery Phase of IRI
The extent of interstitial fibrosis was evaluated in the recovery phase of AKI by quantitative image analysis. The sham control...
group did not show interstitial fibrosis (0.02% ± 0.005%), whereas significant interstitial fibrosis was seen in IRI control group 14 d after IRI induction. As shown in Fig. 3A and B, there was a marked decrease in the interstitial fibrosis area in the QQc PBMNCs group compared with that in the IRI control group (45.2% ± 1.8% in the IRI control group vs. 21.9% ± 8.0% in the QQc PBMNCs group, P < 0.01). The interstitial fibrosis area did not differ between the non-QQc PBMNCs group and the IRI control group, but it was significantly larger than that in the QQc PBMNC group (P < 0.01).

**Improvement of PTC Loss by QQc PBMNCs**

Figure 4A demonstrates the PTCs with anti-mouse CD31 antibody stains of kidney sections. Severe PTC loss was seen in the IRI control group, whereas there was less PTC loss in the QQc PBMNCs group. The time courses of PTC loss in the IRI control group, QQc PBMNCs group, and non-QQc PBMNCs group are shown in Fig. 4B. Forty-eight hours after induction of IRI, there was significantly less PTC loss in the QQc PBMNCs group than in the IRI control group or the non-QQc PBMNCs group (IRI control: 43.1% ± 2.0%; QQc PBMNCs: 21.5% ± 1.0%; and non-QQc PBMNCs: 78.4% ± 2.3%). Thus, injection of QQc PBMNCs had a protective effect against PTC loss in the early phase of IRI.

**Homed Human PBMNCs in Mouse Kidneys Express Mouse Endothelial Antigen**

We evaluated whether human PBMNCs in mouse kidneys express vascular endothelial antigen. QQc PBMNCs did not express CD31 24 h after injection, but some QQc PBMNCs expressed CD31 14 d after induction of IRI (Fig. 5).

**Homing of Injected Human Cells to the Kidney and Other Organs**

Recruitment of human PBMNCs to the kidney was investigated through tracking PKH-labeled cells by immunofluorescence microscopy.

In a preliminary examination, only 0.2 to 2 PBMNCs (per section) were found in IRI kidneys at 24 h after injection with $5 \times 10^6$ QQc PBMNCs. As for $1 \times 10^6$ QQc PBMNCs, only 2 to 3 QQc PBMNCs (per section) were found in the kidneys 24 h after cell injection in the sham operation control group. However, significant accumulation of PBMNCs in IRI kidneys was found after injection with $1 \times 10^6$ QQc PBMNCs as shown in Fig. 6. Injected QQc PBMNCs were present in the spaces between tubules including in the spaces around capillaries. Homing of QQc PBMNCs in the kidney peaked 24 h after injection and then gradually decreased,
with a few PKH-labeled cells still present 14 d after induction of IRI. More PBMNCs accumulated in IRI kidneys after injection of the non-QQc PBMNCs group than did in those of the QQc PBMNCs group (Table 1). The number of non-QQc PBMNCs in IRI kidneys peaked 48 h after injection and then gradually decreased.

Recruitment of injected human PBMNCs to other organs was also observed (Fig. 7; Table 2), with PBMNCs being detected in the normal lung, spleen, and bone marrow of each group. In each of these organs, accumulation of non-QQc PBMNCs was greater than that of QQc PBMNCs.

**Effect of QQc Culture on EPC-CFU and Cell Population**

The effect of QQc culture on PBMNCs was evaluated by EPC-CFU assay and FCM analysis. QQc culture of PBMNCs from healthy volunteers significantly increased the number of definite and total EPC colonies compared with uncultured PBMNCs \((P < 0.05; \text{Fig. 8})\). Cell population analysis by FCM showed that 1 wk of QQc culture significantly increased the number of EPC marker-positive cells (CD34\(^+\) cells and CD133\(^+\) cells) and anti-inflammatory M2 macrophage marker-positive cells (CD206\(^+\) cells). As shown in Fig. 9A, there were only a few cells in gate C among the non-QQc PBMNCs, but 1 wk of QQc culture significantly increased the cells in gate C (almost all of which were M2 macrophages). CD34\(^+\) cells initially existed in gate A (lymphocyte fraction) and increased in gate B (monocyte fraction) after QQc culture. The ratio of the percentage positive cell population between the total cells of QQc PBMNCs and non-QQc PBMNCs is shown in Fig. 9B.

**Discussion**

We found that human PBMNCs cultured in a vasculogenic conditioning medium dramatically improved renal function and reduced histological damage in a mouse model of IRI-induced AKI, even when administered 24 h after induction of AKI. BUN and Cr levels were significantly reduced 48 h
after injection of QQc PBMNCs and were significantly lower than those in the IRI control and non-QQc PBMNCs groups. Both acute PTC loss and interstitial fibrosis in the recovery phase of IRI were also significantly reduced in the QQc PBMNCs group compared with those in the IRI control and the non-QQc PBMNCs groups. Asahara et al. isolated

Table 1. Homing of Injected Human PBMNCs in the Kidney.

| Time after Cell Injection | IRI Model |     |     |     | Sham |
|--------------------------|-----------|-----|-----|-----|------|
| QQc PBMNCs group         | 24 h      | 48 h| 7 d | 14 d| 24 h |
|                         | 86.3 ± 6.4| 44.1± 3.4| 40.9± 6.8| 50.8± 6.3| 1.2 ± 0.8 |
| Non-QQc PBMNCs group     | 229.0 ± 16.0*| 350.3± 25.2*| 150.9± 35.2*| 40.9± 5.1| ND   |

Note: Data indicate PBMNCs' number in each organ slice in 3 mice at indicated time point.
Abbreviation: PBMNC, peripheral blood mononuclear cell; IRI, ischemia reperfusion injury; QQc, quality and quantity control; ND, not done.
*P < 0.01 versus post-QQc group at same time point.
CD34+ EPCs from peripheral blood MNCs, and CD34+ EPCs dramatically improved hind-limb ischemia in an animal model. In this experiment, we proved that human QQc PBMNCs can ameliorate kidney organ damage due to IRI. These findings might encourage the clinical application of cell-based therapy for AKI, which still has no established treatment.

After injection with human QQc PBMNCs, some of these cells were localized in the space between the tubules in the early phase of IRI-induced AKI. Although many cells were trapped in the lungs, bone marrow, and spleen after injection via the tail vein, a sufficient number reached the kidney and influenced the progression of kidney damage. Although the same number of QQc PBMNCs (1 × 10^6 cells) was injected in the sham control group, almost none of these cells were found in the kidney. Our preliminary experiment revealed that the number of PBMNCs used for cell therapy had an important influence on the effect. When we injected 5 × 10^4 QQc PBMNCs 24 h after induction of IRI, almost none of the injected cells were recruited to the kidneys and neither renal function nor pathological findings improved compared with those in the IRI control group. Therefore, both a sufficient number of cells and ischemic signaling may be necessary for recruitment of MNCs to a damaged organ. In addition, more non-QQc PBMNCs than QQc PBMNCs were trapped in several organs including the lungs, spleen, and bone marrow.

Table 2. Homing of Injected Human PBMNCs in Lung, Spleen, and Bone Marrow.

| Time after Cell Injection | IRI Model | Sham |
|--------------------------|-----------|------|
|                          | 24 h      | 48 h | 7 d | 24 h |
| Lung                     |           |      |     |      |
| QQc PBMNCs group         | 38.9 ± 9.9| 9.3 ± 1.3 | 13.4 ± 2.7 | 11.3 ± 2.3 |
| Non-QQc PBMNCs group     | 140.5 ± 15.9* | 133.3 ± 15.9* | 14.8 ± 4.6* | ND |
| Spleen                   |           |      |     |      |
| QQc PBMNCs group         | 157.8 ± 29.5 | 74.6 ± 15.2 | 28.9 ± 6.0 | 29.3 ± 4.4 |
| Non-QQc PBMNCs group     | 478.3 ± 15.7* | 541.9 ± 20.6* | 379.6 ± 87.3* | ND |
| Bone marrow              |           |      |     |      |
| QQc PBMNCs group         | 19.5 ± 3.1 | 2.5 ± 0.8 | 1.3 ± 0.5 | 3.3 ± 1.7 |
| Non-QQc PBMNCs group     | 132.2 ± 3.8* | 140.1 ± 12.1* | 78.8 ± 32.3* | ND |

Abbreviations: PBMNC, peripheral blood mononuclear cell; IRI, ischemia reperfusion injury; QQc, quality and quantity control, ND, not done.

*p < 0.05 vs. QQc PBMNCs group at same time points.

Fig. 7. Homing of quality and quantity control (QQc) peripheral blood mononuclear cells (PBMNCs) into lung, spleen, and bone marrow 24 h after injection of 1 × 10^6 QQc PBMNCs.

Fig. 8. Endothelial progenitor cell (EPC) colony-forming assay. QQc culture significantly increased the number of definite EPC (dEPC) colonies and total EPC colonies. Open bar indicates dEPC and closed bar indicates primitive EPC (pEPC) colonies. Abbreviation: PBMNC, peripheral blood mononuclear cell; QQc, quality and quantity control; EPC, endothelial progenitor cell; CFU, colony-forming unit; ns, not significant. *P < 0.05 between groups.

Ohtake et al
vascular endothelial growth factor receptor-2. * mononuclear cells; QQc, quality and quantity control; VEGFR-2, scatter–area; SSC-A, side scatter–area; PBMNCs, peripheral blood

bone marrow. QQc PBMNCs might have more potential than non-QQc PBMNCs in targeting damaged organs in response to ischemic signals. However, this has not been determined, and further studies are necessary to evaluate whether selective targeting of damaged organs by human PBMNCs is influenced by QQc culture.

In the first 7 d after IRI, human QQc PBMNCs recruited to the kidneys did not express endothelial antigen markers. Therefore, it is conceivable that human PBMNCs mediated renal repair in the early phase of IRI by paracrine mechanisms rather than by replacement of damaged vessels. Local production of various cytokines by QQc PBMNCs, including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) 1, and angiopoietins, might be involved in promoting cellular repair in this model. The therapeutic effect occurred despite a relatively small number of injected cells and their short-term residence in the target zone. Therefore, mechanisms other than transdifferentiation of injected cells delivered to the damaged organs into tissue-specific cells might play a significant role in the observed positive outcome. Normal human CD34+ cells not only express transcripts for but also secrete detectable amounts of VEGF, HGF, IGF-1, fibroblast growth factor 2, Flt-3 ligand, and IL 825. Sahoo et al. demonstrated that the exosomes secreted from mobilized human CD34+ cells had angiogenic paracrine activity in vitro and in vivo26. Masuda et al. demonstrated enhanced gene expression for vascular regeneration and anti-inflammation in QQc PBMNCs using quantitative real-time polymerase chain reaction (qRT-PCR)19. Gene expression of proangiogenic growth factors (VEGF, angiopoietins, and IGF-1) and proangiogenic cytokines (IL-8 and IL-10) was significantly higher, and inflammatory cytokines were significantly lower in QQc PBMNCs than in non-QQc PBMNCs. They expanded their in vitro findings to an in vivo qRT-PCR experiment (gene expression for tissue regeneration in murine ischemic muscle) and confirmed the mechanism by which the cell therapy ameliorated organ damage. Although we could not perform gene expression experiments to examine tissue regeneration in the damaged kidneys, the regenerative paracrine activity of QQc PBMNCs might be related to the mechanisms that reduced tissue damage and improved function in our study. Fourteen days after IRI, a small number of QQc PBMNCs showed expression of CD31 antigen. Therefore, a few of the injected human QQc PBMNCs might have collaborated with resident mouse endothelial cells to restore PTC damage in the recovery phase of IRI-induced AKI.

QQc culture influenced the effects of human PBMNCs; since non-QQc PBMNCs did not improve IRI as did QQc PBMNCs, despite the same number of cells being injected. In fact, some pathological parameters (including cast formation and loss of the brush border) were worse in the non-QQc PBMNCs group compared with the IRI control group. On the other hand, QQc PBMNCs dramatically improved IRI in the present study. The reasons for this difference in potential for ameliorating kidney damage between non-QQc PBMNCs and QQc PBMNCs should be examined.

We found a significant difference in EPC colony-forming potential (vasculogenic potential) between non-QQc PBMNCs and QQc PBMNCs, with the latter showing more potential to form EPC colonies. According to a previous report17, non-QQc PBMNCs and QQc PBMNCs had different regenerative potentials. The total cell count decreased during QQc culture, but there was a significant increase in CD34+ cells and CD133+ cells. Furthermore, QQc culture induced macrophages that were phenotypically polarized into angiogenic, anti-inflammatory subsets: classical M1 to alternative CD206+ M2. qRT-PCR revealed increased expression of proangiogenic genes in QQc-PBMNCs compared to that in non-QQc PBMNCs19. Expansion of vasculogenic CD34+ cells and phenotypic transition of MNCs with anti-inflammatory and angiogenic potential was also confirmed in this study and might be important for in vivo application of this cell therapy.
The decrease of BUN, serum Cr, and corresponding injury parameters in QQc PBMCNs groups was rapid and dramatic. These findings suggest that protection could have been largely due to rapid improvement in renal blood flow (RBF). RBF is the major factor that influences renal injury and function in the context of early AKI. In turn, improvement of RBF could be due to protection from vasoconstriction and/or protection from endothelial injury or loss. However, we could not determine whether the protective effect was due to the primary vasomotor effects or primary endothelial effects. Furthermore, rapid improvement in RBF and the consequent relief from hypoxia/ischemia will protect the endothelium from ongoing damage. We could not precisely determine the pathophysiological mechanisms; however, from the results in this study, we postulate that protection from continuing vasoconstriction and endothelial damage are possible mechanisms of cell therapy in the IRI-induced AKI model. Cytokines released by QQc PBMCNs might improve RBF or stimulate regeneration of surviving mouse endothelial cells. By relieving persistent ischemia, cell therapy could have decreased overall injury, thereby decreasing long-term injury by tubulointerstitial fibrosis. Even delayed administration of human QQc-cultured PBMCNs provided protection against continued acute injury.

In summary, human PBMCNs cultured in a vasculogenic conditioning medium dramatically improved IRI-induced AKI in mice, even when administered 24 h after induction of AKI. The amelioration of PTC damage by QQc PBMCNs resulted in significant improvement of interstitial fibrosis during the recovery phase of IRI. Microcirculatory improvement and anti-inflammatory mechanisms mediating significant changes of cell populations to cells that possess the nature of EPCs and anti-inflammatory macrophages might contribute to the improvement of IRI-induced AKI in this model. Non-QQc human PBMCNs did not improve IRI-induced AKI in this model. Therefore, whole PBMCNs might not be appropriate for cell therapy. The phenotypic transition of PBMCNs to cells with regenerative and anti-inflammatory potential might be required for effective cell therapy. More direct mechanisms why the injured kidney tissues and functions were improved by cell therapy should be clarified. However, the fact that human-cultured PBMCNs could improve severe AKI in mice might be an important step and open the next door of this cell therapy to clinical application.

**Authors’ Note**

All authors gave consent for submission and publication.

**Acknowledgments**

We deeply thank Dr. Takao Suzuki, the president of Tokushukai Medical Group, for his continuous and unifying support for this project. We also thank Mrs. Kayo Arita and Mr. Satoshi Fujimura, Department of Plastic and Reconstructive Surgery, Juntendo University School of Medicine, Ochanomizu, Japan, for their technical assistance.

**Author Contribution**

The authors T.O., S.K., and R.T. made the experimental protocol, and T.O. conducted the whole experiments. The authors Y.M., K.I., H.M., and S.H. supported the experiments; R.M., M.S., D.K., and E.N. supported to establish the IRI model and creatinine measurement; K.O. supported pathological experiments including tissue fixation, preparation of tissue specimen, and immunofluorescent staining; S.S. reviewed the manuscript and advised to make the final manuscript; and H.M. permitted the experiment.

**Ethics Approval and Consent to Participate**

Ethics approval was obtained before starting the study. The approval number of the ethical committee was written in the manuscript. The mouse experimental protocol was approved by the Center for Animal Research at Juntendo University.

**Statement of Human and Animal Rights**

Human rights and privacy were fully protected in this study. Animal care and treatment conformed to institutional guidelines and international laws and politics.

**Statement of Informed Consent**

Peripheral blood from healthy volunteers were obtained after giving oral and written informed consent.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: All experiments were conducted using a research grant from Shonan Kamakura General Hospital.

**References**

1. Santos WJ, Zanetta DM, Pires AC, Lobo SM, Lima EQ, Burdmann EA. Patients with ischemic, mixed and nephrotoxic acute tubular necrosis in the intensive care unit—a homogeneous population? Crit Care. 2006;10(2):R68.

2. Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, Schetz M, Tan I, Bouman C, Macedo E, Gibney N, Tolwani A, Ronco C; Beginning and Ending Supportive Therapy for the Kidney (BEST Kidney) Investigators. Acute renal failure in critically ill patients: a multinational, multicenter study. JAMA. 2005;294(7):813–818.

3. Li B, Cohen A, Hudson EH, Motlagh D, Amrani DL, Duffield JS. Mobilized human hematopoietic stem/progenitor cells promote kidney repair after ischemia/reperfusion injury. Circulation. 2010;121(20):2211–2220.

4. La Manna G, Bianchi F, Cappuccilli M, Cenacchi G, Tarantino L, Pasquinelli G, Valente S, Della Bella E, Cantoni S, Claudia C, Neri F, Tsivian M, Nardo B, Ventura C, Stefoni S. Mesenchymal stem cells in renal function recovery after acute kidney injury: use of a differentiating agent in a rat model. Cell Transplant. 2011;20(8):1193–1208.
5. Eliopoulos N, Zhao J, Forner K, Birman E, Young YK, Bou- 
chentour M. Erythropoietin gene-enhanced marrow mesench- 
ymal stromal cells decrease cisplatin-induced kidney injury 
and improve survival of allogeneic mice. Mol Ther. 2011; 
19(11):2072–2083.

6. Milwid JM, Ichimura T, Li M, Jiao Y, Lee J, Yarmush JS, 
Parekkadan B, Tilles AW, Bonventre JV, Yarmush ML. 
Secreted factors from bone marrow stromal cells upregulate 
IL-10 and reverse acute kidney injury. Stem Cells Int. 2012; 
2012:392050.

7. Liu P, Feng Y, Dong C, Yang D, Li B, Chen X, Zhang Z, Wang 
Y, Zhou Y, Zhao L. Administration of BMSCs with muscone 
in rats with gentamicin-ALI improves their therapeutic 
efficacy. PLoS One. 2014;9(5):e97123.

8. Moghadasali R, Azarmina M, Hajarisrollah M, Arghani H, Nas- 
siri SM, Molazem M, Vossough A, Mohtimafi S, Niaiarasi M, 
Ajdari Z, Yazdi RS, Bagheri M, Ghaanati H, Rafiei B, Gheisari 
Y, Baharvand H, Aghdami N. Intra-renal arterial injection of 
autologous bone marrow mesenchymal stromal cells amelio-
rates cisplatin-induced acute kidney injury in a rhesus macaque 
mulatta monkey model. Cytotherapy. 2014;16(6):734–749.

9. Yasuda K, Ozaki T, Saka Y, Yamamoto T, Gotoh M, Ito Y, 
Yuzawa Y, Matsu S, Maruyama S. Autologous cell therapy 
for cisplatin-induced acute kidney injury by using non-
expanded adipose tissue-derived cells. Cytotherapy. 2012; 
14(9):1089–1100.

10. Feng Z, Ting J, Alfonso Z, Streem BM, Fraser JK, Rutenberg J, 
Huo HC, Pinkemell K. Fresh and cryopreserved, uncultured 
adipose tissue-derived stem and regenerative cells ameliorate 
ischemia-reperfusion-induced acute kidney injury. Nephrol 
Dial Transplant. 2010;25(10):3874–3884.

11. Burgos-Silva M, Semedo-Kuriki P, Donizetti-Oliveira C, 
Costa PB, Cenedeze MA, Hiyane MI, Pacheco-Silva A, 
Camara NO. Adipose tissue-derived stem cells reduce acute 
and chronic kidney damage in mice. PLoS One. 2015;10(11): 
e0142183.

12. Overath JM, Gauer S, Obermuller N, Schubert R, Schafer R, 
Gesiger H, Baer PC. Short-term preconditioning enhances the 
therapeutic potential of adipose-derived stromal/stem cell-
conditioned medium in cisplatin-induced acute kidney injury. 
Exp Cell Res. 2016;342(2):175–183.

13. Morigi M, Rota C, Montemurro T, Montelatici E, Lo Cicero V, 
Imberti B, Abbate M, Zoja C, Cassis P, Longaretti L, Rebulla 
P, Introna M, Capelli C, Benigni A, remuzzi G, Lazzari L. Life-
sparing effect of human cord blood-mesenchymal stem cells in 
experimental acute kidney injury. Stem Cells. 2010;28(3): 
513–522.

14. Liu P, Feng Y, Dong D, Liu X, Chen Y, Wang Y, Zhou Y. 
Enhanced renoprotective effect of IGF-1 modified human umbil- 
cical cord-derived mesenchymal stem cells on gentamicin-
induced acute kidney injury. Sci Rep. 2016;6:20287.

15. Rota C, Imberti B, Pozzobon M, Piccoli M, De Coppi P, Atala 
A, Gagliardi E, Xinaris C, Benedetti V, Fabricio AS, Squar- 
cina E, Abbate M, Benigni A, Remuzzi G, Morigi M. Human 
amniotic fluid stem cell preconditioning improves their 
regenerative potential. Stem Cells Dev. 2012;21(11):1911–1923.

16. Toyohara T, Mae S, Sueta S, Inoue T, Yamaguchi Y, Kawamoto 
T, Kasahara T, Hoshina A, Toyota T, Tanaka H, Araksa 
T, Sato-Otsubo A, Takahashi K, Sato Y, Yamaji N, Ogawa S, 
Yamanaka S, Osafune K. Cell therapy using human induced 
pluripotent stem cell-derived renal progenitors ameliorates 
acute kidney injury in mice. Stem Cells Transl Med. 2015; 
4(9):980–992.

17. Masuda H, Iwasaki H, Kawamoto A, Akimarua H, Ishikawa M, 
Li M, Shizuno T, Sato A, Ito R, Horii M, Ishida H, Kato S, 
Asahara T. Development of serum-free quality and quantity 
control culture of colony-forming endothelial progenitor cell 
for vasculogenesis. Stem Cells Transl Med. 2012;1(2): 
160–171.

18. Tanaka R, Vaynburg M, Masuda H, Ito R, Kobori M, Miyasaka M, 
Mizuno H, Warren SM, Asahara T. Quality control system 
restores diabetic endothelial progenitor cell vasculogenesis 
and accelerates wound closure. Diabetes. 2013;62(9):3207–3217.

19. Masuda H, Tanaka R, Fujimura S, Ishikawa M, Akimarua H, 
Shizuno T, Sato A, Okada Y, Iida Y, Itoh I, Itoh Y, Kamiguchi 
H, Kawamoto A, Asahara T. Vasculogenic conditioning of 
peripheral blood mononuclear cells promotes endothelial pro-
genitor cell expansion and phenotype transition of anti-
flammatory macrophage and T lymphocyte to cells with 
regenerative potential. J Am Heart Assoc. 2014;3(3):e000743.

20. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, 
Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of 
putative progenitor endothelial cells for angiogenesis. Science. 
1997;275(5302):964–967.

21. Wei Q, Dong Z. Mouse model of ischemic acute kidney injury: 
technical notes and tricks. Am J Physiol Renal Physiol. 2012; 
303(11):F1487–F1494.

22. Yuen PST, Dunn SR, Miyaji T, Yasuda H, Sharma K, Star RA. 
A simplified method for HPLC determination of creatinine in 
mouse serum. Am J Physiol Renal Physiol. 2004;286(6): 
F1116–F1119.

23. Stockman G, Leemans JC, Claessen N, Weening JF, Florquin S. 
Hematopoietic stem cell mobilization therapy accelerates 
recovery of renal function independent of stem cell contribu-
tion. J Am Soc Nephrol. 2005;16(6):1684–1692.

24. Hosoya A, Hoshi K, Sahara N, Ninomiya T, Akahane S, Kawamoto 
T, Ozawa H. Effects of fixation and decalcification on the 
immunohistochemical localization of bone matrix proteins in 
fresh-frozen bone sections. Histochem Cell Biol. 2005;123(6): 
639–646.

25. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, 
Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, 
Ratajczak MZ. Numerous growth factors, cytokines, and che-
mosines are secreted by human CD34(+) cells, myeloblasts, 
erthoblasts, and megakaryoblasts and regulate normal hema-
topoiesis in an autocrine/paracrine manner. Blood. 2001; 
97(10):3075–3085.

26. Sahoo S, Klychko E, Thorne T, Misener S, Shinnick K, Millay 
M, Ito A, Liu T, Kamide C, Agarwal H, Perlman H, Qin G, 
Kishore R, Losordo DW. Exosomes from human CD34+ stem 
cells mediate their proangiogenic paracrine activity. Circ Res. 
2011;109(7):724–728.