Mesenchymal stromal cells are retained in the renal cortex independently of their metabolic state after renal intra-arterial infusion

Jesus M. Sierra-Parraga[1,2], Anders Munk[3], Christine Andersen[3], Stine Lohmann[2,3], Cyril Moers[4], Carla C. Baan[1], Rutger J. Ploeg[5], Merel Pool[4], Anna K. Keller[2], Bjarne K. Møller[6], Henri Leuvenink[4], Martin J. Hoogduijn[1], Bente Jespersen[3], Marco Eijken[3,6]

[1]Internal Medicine Department, University Medical Center Rotterdam, Erasmus MC, Rotterdam, the Netherlands;

[2]Department of Renal Medicine, Aarhus University Hospital, Aarhus, Denmark;

[3]Institute of Clinical Medicine, Aarhus University, Aarhus, Denmark;

[4]Department of Surgery – Organ Donation and Transplantation, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands;

[5]Nuffield Department of Surgical Sciences and Oxford Biomedical Research Centre, University of Oxford, Oxford, UK;

[6]Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark.

Jesus Maria Sierra Parraga - Nephrology and Transplantation, Dept. of Internal Medicine, Erasmus MC, University Medical Center, Rotterdam, the Netherlands j.sierraparraga@erasmusmc.nl

Anders Munk - Department of Clinical Medicine - Department of Medicine and Nephrology C, Aarhus University, Aarhus, Denmark amunk@clin.au.dk

Christine Schmidt Andersen - Department of Clinical Medicine - Department of Urology chscad@clin.au.dk

Stine Lohmann - Department of Clinical Medicine - Department of Medicine and Nephrology C, Aarhus University, Aarhus, Denmark stiloh@clin.au.dk

Cyril Moers - Department of Surgery – Organ Donation and Transplantation, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands c.moers@umcg.nl

Carla Baan - Nephrology and Transplantation, Dept. of Internal Medicine, Erasmus MC, University Medical Center, Rotterdam, the Netherlands c.c.baan@erasmusmc.nl

Rutger Ploeg - Nuffield Department of Surgical Sciences and Oxford Biomedical Research Centre, University of Oxford, UK rutger.ploeg@nds.ox.ac.uk

Merel Pool - Department of Surgery – Organ Donation and Transplantation, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands m.b.f.pool@umcg.nl

Anna Krarup Keller - Department of Clinical Medicine - Kidney Diseases, Aarhus University, Aarhus, Denmark anna.keller@clin.au.dk

Bjarne K. Møller - Department of Clinical Immunology, Aarhus University Hospital, Denmark bjamoell@rm.dk

Henri Leuvenink - Department of Surgery – Organ Donation and Transplantation, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands h.g.d.leuvenink@umcg.nl

Martin Hoogduijn - Nephrology and Transplantation, Dept. of Internal Medicine, Erasmus MC, University Medical Center, Rotterdam, the Netherlands m.hoogduijn@erasmusmc.nl

Bente Jespersen - Department of Renal Medicine, Aarhus University Hospital, Aarhus, Denmark bente.jespersen@clin.au.dk

Marco Eijken - Institute of Clinical Medicine, Aarhus University, Aarhus, Denmark. m.eijken@clin.au.dk

Key words: Mesenchymal stromal cells, renal intra-arterial, cell therapy, ischemia reperfusion injury, porcine model.

Running title: Passive retention of MSC after renal intra-arterial infusion.

Corresponding author

J.M. Sierra Parraga

Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands

Postal address: Postbus 2040, 3000 CA Rotterdam

Phone number: 0034-691400048

E-mail: j.sierraparraga@erasmusmc.nl

Abbreviations

DPBS Dulbecco's phosphate buffered saline

HI-MSC Heat-inactivated mesenchymal stromal cells

IV Intravenous

MSC Mesenchymal stromal cells

Qdot 655 Quantum dots 655

qPCR Quantitative polymerase chain reaction

Abstract

The regenerative capacities of mesenchymal stromal cells (MSC) make them suitable for renal regenerative therapy. The most common delivery route of MSC is via intravenous infusion, which is associated with off-target distribution. Renal intra-arterial delivery offers a targeted therapy but limited knowledge is available regarding the fate of MSC delivered via this route. Therefore, we studied the efficiency and tissue distribution of MSC after renal intra-arterial delivery to a porcine renal ischemia reperfusion model. MSC were isolated from adipose tissue of healthy male pigs, fluorescently labelled and infused into the renal artery of female pigs. Flow cytometry allowed MSC detection and quantification in tissue and blood. In addition, qPCR was used to trace MSC by their Y-chromosome. During infusion, a minor number of MSC left the kidney via the renal vein and no MSC were identified in arterial blood. Ischemic and healthy renal tissue were analyzed 30 minutes and 8 hours after infusion and 1-4 x 10^4 MSC per gram of tissue were detected, predominantly, in the renal cortex, with a viability greater than 70%. Confocal microscopy demonstrated mainly glomerular localization of MSC, but they were also observed in the capillary network around tubuli. The infusion of heat inactivated (HI)-MSC, which are metabolically inactive, through the renal artery showed that HI-MSC were distributed in the kidney in a similar manner as regular MSC, suggesting a passive retention mechanism. Long term MSC survival was analyzed by Y-chromosome tracing and demonstrated that a low percentage of the infused MSC were present in the kidney 14 days after administration, while HI-MSC were completely undetectable. In conclusion, renal intra-arterial MSC infusion limited off-target engraftment, leading to efficient MSC delivery to the kidney, most of them being cleared within 14 days. MSC retention was independent of the metabolic state of MSC, indicating a passive mechanism.

Introduction

Mesenchymal stromal cells (MSC) have regenerative properties which induce tissue regeneration in the injured kidney [1-3]. MSC secrete a variety of cytokines and growth factors that stimulate endothelial cell proliferation, enhance angiogenesis and reduce endothelium permeability [4-7]. Moreover, MSC are able to reduce inflammation through the secretion of immunoregulatory mediators and induce anti-inflammatory M2 macrophages [8-10].

Studies in rodents have shown that MSC can improve renal function in a transplant model [11] and restore renal structure and function in an acute kidney injury model [12]. A swine renal artery stenosis model showed that MSC are able to reduce fibrosis and inflammation of the renal medulla [13]. Moreover, in a porcine transplant model MSC treatment improved glomerular and tubular functions and protected the kidney from fibrosis [14].

As a result, several clinical trials are now trying to translate this success into an effective MSC therapy [15]. In these trials, the safety and efficacy of intravenous (IV) MSC infusion were investigated as a treatment for different renal diseases and to improve the outcome of kidney transplantation [2]. In the aforementioned animal and human studies, IV infusion of MSC was proven to be an easy and safe administration route. However, IV delivery of MSC has been shown to have some limitations. IV infused MSC do not specifically target the injured organ [16], which could lead to unwanted off-site effects. It has been shown that IV infusion of MSC leads to entrapment of MSC in the lung microvasculature, from where they are rapidly cleared by the immune system [17-19].

Intra-arterial infusion is a promising option to deliver MSC specifically to injured organs which increases MSC delivery efficiency compared to IV infusion [20,21]. This was demonstrated in a rat kidney transplantation model [22] and in kidney injury rat models of polycystic kidney disease [23] and glomerulonephritis [24]. Studies in an ovine model showed that administration of MSC

through the renal artery leads to their engraftment in glomerular and tubular capillaries [25]. In swine models, renal intra-arterial delivery of MSC has been shown to reduce inflammation and fibrosis and improved revascularization, restoring renal function [13,26]. In an acute kidney injury monkey model, MSC infused through the renal artery were found in glomeruli and tubuli as well, and were able to restore renal function [27,28]. Moreover, a phase 1/2A human clinical trial has been carried out using renal intra-arterial infusion of autologous MSC treatment without reporting any adverse effects [29].

Albeit renal intra-arterial MSC delivery is deemed feasible, the efficiency of MSC delivery is not known, which makes it difficult to study dose-dependent effects. In addition, the mechanisms behind the retention of MSC are unknown. To be able to better correlate renal intra-arterial based MSC therapy with observed effects in clinical and preclinical studies, more detailed knowledge regarding MSC retention and localization is crucial. These key questions require answering to further understand the role of exogenously administered MSC to the injured kidney and to explore the therapeutic use of MSC for kidney repair.

In the present study renal intra-arterial MSC infusion was investigated in a porcine ischemia-reperfusion injury kidney model. This model allows us to study MSC delivery in human sized kidneys making it translatable to studies in humans. The goal of the study was to determine the efficiency and tissue distribution of MSC delivered via the renal artery as well as to elucidate the mechanism responsible for MSC retention and survival in the kidney after infusion.

Materials and Methods

Institutional regulations

Female pigs of Danish Landrace and Yorkshire crossbreed weighing 40 kg were used. Animal care and experiments followed guidelines by the European Union (directive 2010/63/EU) and local

regulations. The Animal Experiments Inspectorate approved the study (reference-number 2013−15−2934−00925 and 2017-15-0201-01367).

Isolation and expansion of MSC

Subcutaneous adipose tissue was collected from healthy 40-60 kg male Danish landrace pigs during surgery as a waste product. In total, 4 g fat was cut in small pieces and washed twice with 30 mL Dulbecco's phosphate buffered saline (DPBS) (ThermoFisher, Manhattan, NY, USA) by centrifugation at 850g for 5 min. Adipose tissue was dissociated in 10 mL RPMI-1640 medium (ThermoFisher) containing 150 U/mL collagenase type IV (ThermoFisher) in a GentleMACS Octo tissue dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) using protocol 37C-mr-ATDK-1. After dissociation, 10 mL culture medium was added and cells were pelleted at 650g for 10 min. Pellet was resuspended in 10 mL culture medium and filtered through a 70 µm cell strainer and seeded in culture flasks. Cells were cultured at 37°C and 5% CO_2 until they reached 80-90% confluency (about 7 days). Culture medium was replaced twice a week and MSC were sub-cultured until passage 3 before use or cryostored in 50% serum, 10% DMSO in liquid N_2. MSC culture medium consisted of minimum essential medium (Sigma-Aldrich) supplemented with 15% fetal bovine serum (Sigma-Aldrich), 50 U/ml penicillin + 50 µg/ml streptomycin (ThermoFisher), and 2 mM L-glutamine (ThermoFisher).

MSC characterization

Morphology of MSC was observed with an axiovert 40 C microscope (Zeiss, Oberkochen, Germany) coupled to a Zeiss CanonSLR camera (Zeiss) and their fibroblastic appearance was confirmed (Figure 1A). MSC (p3) were characterized by the expression and absence of specific membrane markers. About 1 x 10^5 MSC in 100 µL DPBS were incubated for 30 minutes with 1 µL anti-human CD29 (APC, catalog #17-0299-42, Biolegend), 1µL CD44 (PE, catalog #17-0441-81, Biolegend), 1µL CD90 (BV421, catalog #561557, Biolegend), which are described to cross-react

with swine species, and the absence of negative membrane markers was assessed with 5 µL anti-pig CD31 (FITC, catalog #MCA1746F, Bio-rad, Hercules, CA, USA) and 5 µL anti-pig CD45 (FITC, catalog #MCA1222F, Bio-rad). Next, cells were analyzed by multiparameter flow cytometry using a Novocyte flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA). All used MSC batches were >95% positive for CD29, CD44, CD90 and negative for CD31 and CD45 (Figure 1B and C). MSC size was measured directly after trypsinisation. MSC in suspension were transferred to a Burker-Turk counting chamber (ThermoFisher) and pictures were made under 100x magnification and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA) using plugins ij_Geodesics and Cell Magic Wand. The grid of the counting chamber was used as a size reference. For each cell the, maximum and minimum diameter was determined and the average diameter was used to express cell diameter. For each MSC batch cell diameter was determined of at least 100 cells. The size of infused MSC ranged from 10 to 30 µm and the median was 16-18 µm (Figure 1D and E).

MSC labelling

MSC were cultured until 90% confluency, trypsinized and labelled with Quantum dots (Qdot) 655 (ThermoFisher) using 1,5 µL of the reagent per million MSC according to manufacturer's protocol immediately before the infusion. Fluorescence was measured prior to each infusion to establish the gating strategy to identify the MSC. For labelling with PKH-26 (Sigma), MSC were cultured as mentioned, trypsinized and labelling was performed following the manufacturer's protocol.

Heat inactivation of MSC

MSC were heat inactivated as previously described [30]. MSC were resuspended in DPBS (1-2 x 10^6/mL) and incubated at 50° C for 30 minutes and cooled-down in ice for 5 minutes.

MSC administration

MSC were pelleted by centrifugation (440g, 5 min) after trypsinization, heat inactivation or labeling. MSC were resuspended in DPBS and filtered over a 70 µm cell strainer. Before administration, cells were counted and visually inspected under a microscope in order to confirm a single cell solution.

Tissue dissociation

In total, 0.5 grams of renal tissue were cut in small pieces and dissociated using a GentleMACS Octo tissue dissociator (Miltenyi Biotec). Renal cortical tissue was dissociated in 2.5 mL RMPI 1640 (ThermoFisher) supplemented with 100 µL enzyme D, 50 µL enzyme R and 12.5 µL enzyme A of a multi tissue dissociation kit 1 (Miltenyi Biotec) and run with protocol 37C_Multi_B. Renal medulla and lung tissue were dissociated in 2.5 mL buffer X supplemented with 25 µL enzyme P, 25 µL buffer Y, 50 µL enzyme D and 10 µL enzyme A of a multi tissue dissociation kit 2 (Miltenyi Biotec) and run with protocol 37C_Multi_E. After dissociation, 8 mL RPMI-1640 were added and the cell suspension was filtered through a 70 µm cell strainer and centrifuged at 500 g for 7 minutes. Pellets were resuspended in 3 mL culture medium and analyzed immediately or cryo-preserved in 50% FBS, 10% DMSO.

Plastic adherent fraction of dissociated tissue

In total, 500 µL cryo-preserved dissociated tissue was thawed and seeded in a T75 culture flask (Nunclon delta surface; Thermo Scientific) with 10 mL MSC medium and incubated for 1 day at 37°C and 5% CO_2. Medium was fully removed after 24 hours and cells were incubated for an additional 4 hours. Before trypsinization, cells were washed twice with PBS (without calcium and magnesium, ThermoFisher) and detached using 2 ml 0.05% Trypsin-EDTA (ThermoFisher). Cells were pelleted by centrifugation at 440g for 5 minutes and measured by flow cytometry or stored at -20°C for subsequent DNA isolation.

Y-chromosome PCR

DNA was isolated from 10 mg of kidney tissue or from cell pellets from the adherent fraction using a NucleoSpin Tissue DNA isolation kit (MACHEREY-NAGEL, *Düren*, Germany) according to manufacturer's protocol. DNA was eluted in 100 µL water and Y-chromosome was detected by qPCR using primers directed to the male specific repeat (MSR) located on the porcine Y-chromosome as previously done by Gruessner *et al.* [31]. Primers directed to porcine S100C gene were used as a pig DNA control. Primer sequences were as follow: MSR forward 5'-CCA TCG GCC ATT GTT TTC CTG TTC A-3', MSR reverse 5'-CCT CTG TGC CCA CCT GCT CTC TAC A-3', S100C forward 5'-ATG CTG GAA GGG ACG GTA ACA ACA-3', and S100C reverse 5'-GCT CAG CTG CTG TCT TTC ACT CGT-3'. qPCR mix consisted of 0.5 µL DNA, 10 pmol of each primer and 1x KiCStart SybrGreen qPCR ReadyMix (Sigma-Aldrich Life Science) in an final volume of 25 µl. Samples were run in duplicate on a ABI 7300 (Perkin Elmer). The thermocycling program included an initial step of 2 minutes at 50° C. Subsequently 10 minutes at 95° C. Followed by a 40 time repeat two-step cycle consisting 95° C for 15 seconds and 60° C for 1 minute.

Anesthetics and surgical procedure

The pigs were sedated with intramuscular injection of Stresnil® (2.2mg/kg) to allow vein cannulation. Intravenous (IV) administration of Ketamine (6mg/kg) and Midazolam (0.5mg/kg) allowed intubation and ventilation keeping CO_2 between 4.5-5.5 kPa. Anesthesia was maintained using IV administrated Fentanyl (15µg/kg/h) and Propofol (3.5 mg/kg/h) preceded by a bolus of 7.5 µg/kg (Fentanyl) and 1.875 mg/kg (Propofol). A bolus of 1.5 L Ringer Acetate was administrated within the first one and a half hours followed by a continuous infusion rate of 400 mL/h to maintain normal hydration and a mean arterial blood pressure ≥60 mmHg in all pigs. Introducers were inserted in the common carotid artery, external jugular vein and femoral artery (Radifocus® Introducer II, Terumo Europe, Leuven, Belgium). The experiments were conducted with or without renal ischemia.

MSC infusion during open surgery

The data presented in figures 2-4 were obtained by an open surgery procedure. Following a midline incision and retroperitoneal exposure of the left kidney, a catheter was positioned in the aorta over a guidewire in the femoral sheet. The catheter was further inserted into the left renal artery where positioning was controlled by palpation. Ischemic injury was performed by clamping the renal artery and vein for 60 minutes. After removing the clamps or directly after catheterization of healthy kidneys, 10 million MSC suspended in 25 mL DPBS were infused into the renal artery followed by a 5 mL DPBS flush both at a rate of 150 mL/h. In both healthy and ischemic kidneys, the renal artery catheter was removed after ten minutes when MSC administration was finished. Blood was collected simultaneously from the carotid artery and renal vein before, during and after MSC infusion. Bilateral nephrectomy was completed after either 30 minutes or 8 hours follow up and the pig was terminated using an IV overdose of pentobarbital (100mg/kg) while in general anesthesia. Both kidneys were weighted and cut longitudinally and horizontally through the medial line after retrieval. Kidney weight ranged from 90 to 110 grams in all pigs. One half of each kidney was used to obtain all tissue material used for analysis and the other one was embedded in PELCO® cryo-embedding compound (Ted Pella, Inc., Redding, CA, USA) and cryopreserved. Each experimental group consisted of 3 pigs.

Non-invasive MSC infusion

The data presented in figures 6 and 7 were obtained by a non-invasive MSC delivery method. Over a guidewire in the femoral introducer sheath, a catheter was introduced in the aorta and contrast agent (Iomeron 350mg/mL, total volume used 40 mL) was administered using X-ray to ensure the correct positioning of the catheter in the renal artery. Hereafter, 10 million MSC in 25 mL DPBS were infused into the renal artery followed by a 5 mL DPBS flush both at a rate of 150 mL/h. The renal artery catheter was removed after ten minutes, when MSC administration was finished.

Bilateral nephrectomy was completed after 30 minutes (n=3) or 14 days follow up (n=8 and n=4 for MSC and heat inactivated (HI)-MSC infused kidneys, respectively) and the pig was terminated using an IV overdose of pentobarbital (100mg/kg) while in general anesthesia. Kidney weight ranged from 90 to 110 grams in all pigs.

MSC detection in kidney and blood by flow cytometry

Blood samples and renal tissue were analyzed using flow cytometry. Whole blood samples drawn from the renal vein and the carotid artery were directly analyzed. From the total dissociated kidney tissue, 400 µL were analyzed by flow cytometry. The fluorescence was measured by excitation with a 405 nm laser and detection with the 660/20 BP, 650 LP filter set in a Novocyte flow cytometer (ACEA Biosciences, Inc., San Diego, CA, USA). To avoid low fluorescent cells from being measured and to speed up the measuring process a fluorescence threshold (median fluorescent intensity = 5000) was applied. Qdot-positive cells found in the cell suspensions were identified as the infused MSC. To confirm that the Qdot-positive cells found in dissociated renal tissue and in whole blood were indeed the infused MSC, simultaneous expression of CD29, CD44 and CD90 and absence of CD31 and CD45 was assessed as mentioned earlier for MSC characterization. The viability of MSC was assessed using the Zombie NIR™ Fixable Viability Kit (Biolegend). From the dissociated renal tissue 100 µL were stained with Zombie NIR™ following the manufacturer's protocol and analyzed by flow cytometry. The total number of MSC in kidney tissue or blood was estimated from the number of events and the volume measured from each sample. Flow cytometry data was analyzed using NovoExpress software (ACEA Biosciences, Inc.).

Confocal microscopy of renal tissue

Kidney biopsies, containing both renal cortex and medulla, of 1 cm^3 formalin-fixed and paraffin-embedded renal tissue biopsies were cut in 3 µm thick slices. Images were obtained using a Leica

SP5 confocal microscope (Leica microsystems, Wetzlar, Germany). Renal tissue auto-fluorescence was used to identify specific kidney structures such as glomeruli and tubules. Qdot 655 signal was detected by exciting the samples at a wavelength of 405 nm and measuring fluorescence emission at 655 nm. Tissue auto-fluorescence was measured at 420 nm to identify different renal structures. Images were analyzed using Fiji from ImageJ.

Cell localization by 3D cryo-imaging

Half kidneys were embedded in mounting medium for cryotomy (PELCO® cryo-embedding compound, Ted Pella Inc.) and frozen in liquid nitrogen. Frozen kidneys were kept at -80° C and shipped to BioInVision (OH, USA). At BioInVision, quantification of engrafted MSC was performed based on detection of fluorescent signal.

Statistical analysis

The Mann-Whitney test was used for comparison of mean numbers of MSC found in renal and lung tissue and MSC found in the blood outflow from the renal vein. The Kruskal-Wallis test was used when more than 2 groups were compared at the same time. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

MSC quantification in blood and tissue

Qdot-labeled MSC showed a high fluorescent signal which allowed clear identification of the cells by flow cytometry (Sup. Figure 1A). To demonstrate the feasibility of a flow cytometry-based detection technique of relatively low numbers of MSC in large amounts of kidney and blood cells, Qdot-labelled MSC were mixed with dissociated renal tissue and whole blood in vitro. Results

showed that Qdot-labeled MSC could be semi quantitatively measured in these samples (Sup. Figure 1B and C).

MSC detection after renal intra-arterial delivery

In total, 10 million Qdot-labelled MSC were infused in vivo through the renal artery to healthy or ischemic kidneys and 30 minutes after administration renal tissue was analyzed for the presence of Qdot-labelled MSC. We observed that MSC were successfully delivered to both healthy and ischemic kidneys (Figure 2A and D; Sup. Figure 3B and C). Measured MSC were in the order of 1-4 x 10^4 MSC per gram of dissociated cortical tissue. To demonstrate that MSC were stably retained in the kidney, the follow-up time of the ischemic group was extended to 8 hours. This demonstrated that the majority of MSC remained in the kidney at least up to 8 hours. The viability of MSC in the kidney tissue measured by flow cytometry ranged from 70 to 80% in all three conditions (Figure 2B). We observed that MSC found in the renal cortex had a bigger size than those found in the renal medulla (Figure 2C).

Qdot-labelled MSC were also detected in dissociated renal medulla, however, MSC numbers were 10-fold lower than those found in the renal cortex. Lung tissue contained numbers 100-fold lower compared to renal cortex, with an average of 200 MSC per gram of tissue (Figure 1D).

During the experiments decribed above, blood samples from the renal vein and carotid artery were drawn to identify the presence of MSC passing through the kidneys. During infusion, an average of 500 MSC per mL were measured leaving the kidney though the renal vein. After the infusion stopped, hardly any MSC were found leaving the kidney (Figure 1D; Sup. Figure 2A). In blood samples from the carotid artery, no MSC were detected either during or after infusion (Sup. Figure 2B). The size of the MSC found in the renal vein outflow was smaller than those retained in the kidney, both in cortical and medullar tissue (Figure 2C).

Qdot-labelled MSC identified in blood and renal tissue were phenotyped to confirm MSC characteristics. Identified MSC expressed CD29, CD90 and CD105 and lacked the expression of CD31 and CD45 (Sup. Figure 4A-D).

Cortical localization of MSC after renal intra-arterial infusion was confirmed by 3D cryo-imaging

3D cryo-imaging was used to confirm the presence and localization of fluorescent MSC in the kidney. Kidneys were collected 30 minutes after administration of 10 million Qdot-labelled MSC and cryo-imaged. In figure 3A, a 3D volume rendering generated from brightfield data from the lateral and medial sides of the kidney are shown. Detected Qdot-positive MSC were pseudocolored in yellow and rendered along with brightfield data. MSC were mainly observed in renal cortical tissue (Figure 3B), supporting the data obtained by flow cytometry.

MSC are located mainly in glomerular structures

Confocal microscopy demonstrated that MSC were localised mostly to glomerular structures in the renal cortex. There was no clear difference regarding the location of MSC observed in healthy and ischemic kidneys. Pictures shown in Figure 4 were taken from healthy kidney tissue. Frequently, several MSC were observed in single glomeruli (Figure 4A-C). The frequency of MSC outside the glomerular structures was much lower. However, MSC could be identified around tubules both in the renal cortex and and medulla (Figure 4C and D, respectively).

MSC stay in the kidney through a passive mechanism

In order to study the mechanism responsible for MSC retention in kidneys, we examined the retention of inactivated MSC in the kidney. HI-MSC were generated by heating MSC to 50°C for 30 minutes, which made them metabolically inactive and therefore they lost their ability to adhere (Figure 5).

Regular MSC or HI-MSC were infused in-vivo via the renal artery to healthy kidneys and MSC presence was assessed 30 minutes after administration by flow cytometry. HI-MSC were retained in similar numbers in the kidney as regular MSC (Figure 6A).

Viability analysis of retained MSC confirmed that regular MSC infused to the kidney remained alive in the renal cortex, whereas infused HI-MSC were indeed non-viable (Figure 6B). Dissociated renal tissue containing MSC was seeded in a culture dish to examine the adherent capacity of MSC in the tissue. Analysis of the adherent fraction of dissociated renal tissue demonstrated the presence of plastic-adherent Qdot-positive MSC, while only background fluorescence was detected in the HI-MSC group (Figure 6C). Confocal microscopy and 3D cryo-imaging of HI-MSC infused kidneys confirmed that HI-MSC were located in the same renal structures as regular MSC (Figure 6D and E, respectively).

The majority of MSC are cleared from the kidney within 2 weeks

Male MSC were administered to female pigs which allowed male MSC tracing by a qPCR of a male specific repeat located on the pig Y-chromosome. First, renal tissue was analyzed for the presence of Y-chromosome in kidneys harvested 30 minutes after infusion of 10 million MSC or HI-MSC. This demonstrated that both MSC and HI-MSC infused kidneys contained high amounts of Y-chromosome 30 minutes after MSC delivery (Figure 7A).

The same experiment was repeated with a 14 days follow up. This showed that Y-chromosome could be detected 14 days after MSC infusion, whereas no such signal was detected in HI-MSC infused kidneys. Albeit Y-chromosome DNA was detected after 14 days, the average relative amount at day 14 was approximately 1% of the amount measured 30 minutes after infusion. Moreover, 3 out of 8 analyzed kidneys showed Y-chromosome DNA signal just above threshold.

Viability of the detected MSC was demonstrated as previously described by analyzing the plastic adherent fraction of the dissociated renal tissue. This showed that dissociated renal tissue isolated

14 days after MSC delivery contained living, plastic-adherent male MSC, whereas no male MSC could be found in the plastic adherent fraction of HI-MSC infused kidneys (Figure 7B).

Discussion

In this study we have tested the efficiency and tissue distribution of in-vivo MSC infusion through the renal artery in a porcine ischemia reperfusion injury model. We showed that targeted MSC delivery via the renal artery is a feasible route to deliver MSC to the kidney. Upon infusion, MSC are distributed throughout the kidney, located mostly in renal cortex and particularly inside glomeruli. MSC are retained in renal tissue presumably through a passive mechanism and after infusion they survive for at least 8 hours. The majority of MSC were cleared from the kidneys within two weeks.

We show that renal intra-arterial targeted infusion of MSC minimizes off-target delivery as only small numbers of MSC leave the kidney during infusion. Previous studies in animal models have simply associated the observed effects with the number of infused, but not delivered, MSC to the injured kidney [25,32,33]. In our study we actually quantify the number of MSC delivered to the target tissue which enables to correlate observed effects with the MSC dose in future studies. In order to do this we used a semi-quantitative flow cytometric method which can be used to quantify MSC delivered to the kidney via renal intra-arterial infusion. Our approach using pre-labelled MSC in combination with flow cytometric analysis of dissociated kidney tissue allowed us to have a good estimate of the absolute amount of MSC that were retained in the kidney. Infusion of 10 million MSC to the kidney resulted in numbers in the order of 1-4 x 10^4 MSC per gram of renal tissue delivered throughout the kidney, although the distribution was not completely homogenous. Extrapolation of measured concentrations of MSC using total kidney weight indicates that several million MSC were delivered per kidney. This number points out that a significant amount of MSC

is not found back in the kidney. However, it should be noted that the measured numbers are a minimum value, as part of the MSC might be lost during the tissue dissociation and analysis process. It is still unknown whether this number of MSC is biologically relevant or therapeutically effective on the kidney. Therefore, our work paves the way for new studies to address this issue.

MSC were retained mostly in glomeruli as demonstrated also in other studies [24,25,32,33]. MSC are relatively large cells, so they might simply get entrapped in the microcapillaries of glomerular structures in a similar manner as MSC are entrapped in the lung's capillary network after intravenous infusion [17]. However, the size of MSC used in our experiments averaged 16 μm which is similar to some white blood cells which are able to pass through the microcapillaries of glomeruli. Red and white blood cells can deform by altering their cytoskeletal structure in order to be able to flow through microcapillaries [34-36]. However, MSC are originally tissue resident cells, which might be a reason not to have the same shape deformation capacities [37,38]. Moreover, HI-MSC were also retained in the same structures. These cells are not metabolically active and therefore unable to deform, which supports our hypothesis of a passive retention mechanism. We have shown that only bigger MSC were found in the renal cortex, while smaller MSC could pass through the glomerular microcapillaries and end up in the renal medulla or even left the kidney. In a rat model, infused MSC stayed in the kidney, whereas other infused cell type, of the same size, failed to stay in renal tissue [24]. This fact suggests that MSC are specifically retained by the kidney itself. In the case that MSC get stuck in the glomerular microcapillaries network, there might be some safety concerns as renal blood flow or function might be compromised. It has been reported in a rat model that after infusion of very high numbers of MSC, parenchymal perfusion decreased and it was restored to normality after 24 hours [32]. Furthermore, several murine pre-clinical studies showed improved function of injured kidneys after delivery of MSC through the renal artery with no adverse effects [23,24,39]. Besides, a human clinical trial have not reported

serious adverse effects upon renal intra-arterial infusion of MSC and even showed, to some extent, improved kidney function [29].

MSC have been shown to be short lived after intravenous infusion [17,18,40]. However, renal intra-arterial infusion of MSC seems to ensure longer survival of MSC after infusion. In our study, MSC viability in the kidney 8 hours after infusion was around 70 to 80%. However, after 14 days, most MSC were cleared from the kidney. Nevertheless, large animal studies have shown the presence of MSCs up to 5 weeks after renal intra-arterial infusion [25], in contrast to our findings. In these large animal models, mostly autologous MSC are used [25-27,33], whereas rodent renal intra-arterial MSC infusion models usually employ allogeneic MSC [21,23,24]. In both cases, similar survival of infused MSC was observed, which suggests that the origin of MSC does not affect survival after delivery. From a logistic and translational point of view, the use of allogeneic MSC is more appealing as large numbers of therapeutic MSC should be readily available for treatment. Our results confirm that infusion of allogeneic MSC is feasible in large animal models and enables survival of MSC in renal tissue.

The mechanism behind MSC retention after renal intra-arterial infusion is, so far, poorly understood. Although we have shown that retention of MSC in the kidney is independent of the metabolic status of the infused MSC, they could be actively attached by the glomerular endothelium. It has been described that MSC can physically interact with endothelial cells via adhesion molecules present in their membrane, such as very late antigen 4 [41,42]. As HI-MSC maintain the proteins expressed on their membrane, they may still be able to interact with the endothelium through this mechanism. The elucidation of the mechanisms behind the effects of MSC is essential to further develop MSC therapies. However, conflicting data found in the literature defend both the paracrine secretion of cytokines [43,44] and the physical interaction with other cells [18,30] as the main mechanism of MSC action. This makes it difficult to draw a conclusion and therefore, additional experimentation is indeed needed.

Summarizing, renal intra-arterially infused MSC are delivered particularly to the glomeruli and survive for at least 8 hours after infusion. Their presence can potentially allow them to interact with injured tissue and elicit a regenerative response. To completely understand the potential of MSC therapy in kidneys, further studies are now starting in order to decipher the specific mechanisms of action of MSC after renal intra-arterial delivery, which will contribute to an improved MSC therapy for treatment of kidney injury.

Acknowledgements

This project is funded by the Lundbeck Foundation under grant application no R198-2015-184 and Fabrikant Vilhelm Pedersen og Hustrus Legat under grant application no 22720 2016.

Author Disclosure Statement

No competing financial interests exist.

References

1. Morigi M, C Rota and G Remuzzi. (2016). Mesenchymal Stem Cells in Kidney Repair. Methods Mol Biol 1416:89-107.
2. Peired AJ, A Sisti and P Romagnani. (2016). Mesenchymal Stem Cell-Based Therapy for Kidney Disease: A Review of Clinical Evidence. Stem Cells Int 2016:4798639.
3. Baraniak PR and TC McDevitt. (2010). Stem cell paracrine actions and tissue regeneration. Regen Med 5:121-43.
4. Morishita R, S Nakamura, Y Nakamura, M Aoki, A Moriguchi, I Kida, Y Yo, K Matsumoto, T Nakamura, J Higaki and T Ogihara. (1997). Potential role of an endothelium-specific growth factor, hepatocyte growth factor, on endothelial damage in diabetes. Diabetes 46:138-42.
5. Nakano N, R Morishita, A Moriguchi, Y Nakamura, SI Hayashi, M Aoki, I Kida, K Matsumoto, T Nakamura, J Higaki and T Ogihara. (1998). Negative regulation of local hepatocyte growth factor expression by angiotensin II and transforming growth factor-beta in blood vessels: potential role of HGF in cardiovascular disease. Hypertension 32:444-51.

6. Yang Y, QH Chen, AR Liu, XP Xu, JB Han and HB Qiu. (2015). Synergism of MSC-secreted HGF and VEGF in stabilising endothelial barrier function upon lipopolysaccharide stimulation via the Rac1 pathway. Stem Cell Res Ther 6:250.
7. Burlacu A, G Grigorescu, AM Rosca, MB Preda and M Simionescu. (2013). Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. Stem Cells Dev 22:643-53.
8. Qi Y, D Jiang, A Sindrilaru, A Stegemann, S Schatz, N Treiber, M Rojewski, H Schrezenmeier, S Vander Beken, M Wlaschek, M Bohm, A Seitz, N Scholz, L Durselen, J Brinckmann, A Ignatius and K Scharffetter-Kochanek. (2014). TSG-6 released from intradermally injected mesenchymal stem cells accelerates wound healing and reduces tissue fibrosis in murine full-thickness skin wounds. J Invest Dermatol 134:526-37.
9. Geng Y, L Zhang, B Fu, J Zhang, Q Hong, J Hu, D Li, C Luo, S Cui, F Zhu and X Chen. (2014). Mesenchymal stem cells ameliorate rhabdomyolysis-induced acute kidney injury via the activation of M2 macrophages. Stem Cell Res Ther 5:80.
10. Bouffi C, C Bony, G Courties, C Jorgensen and D Noel. (2010). IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. PLoS One 5.
11. Iwai S, I Sakonju, S Okano, T Teratani, N Kasahara, S Yokote, T Yokoo and E Kobayash. (2014). Impact of ex vivo administration of mesenchymal stem cells on the function of kidney grafts from cardiac death donors in rat. Transplant Proc 46:1578-84.
12. Morigi M, B Imberti, C Zoja, D Corna, S Tomasoni, M Abbate, D Rottoli, S Angioletti, A Benigni, N Perico, M Alison and G Remuzzi. (2004). Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. J Am Soc Nephrol 15:1794-804.
13. Ebrahimi B, A Eirin, Z Li, XY Zhu, X Zhang, A Lerman, SC Textor and LO Lerman. (2013). Mesenchymal stem cells improve medullary inflammation and fibrosis after revascularization of swine atherosclerotic renal artery stenosis. PLoS One 8:e67474.
14. Baulier E, F Favreau, A Le Corf, C Jayle, F Schneider, JM Goujon, O Feraud, A Bennaceur-Griscelli, T Hauet and AG Turhan. (2014). Amniotic fluid-derived mesenchymal stem cells prevent fibrosis and preserve renal function in a preclinical porcine model of kidney transplantation. Stem Cells Transl Med 3:809-20.
15. Reinders MEJ, C van Kooten, TJ Rabelink and JW de Fijter. (2018). Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. Transplantation 102:35-43.
16. Gao J, JE Dennis, RF Muzic, M Lundberg and AI Caplan. (2001). The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169:12-20.
17. Eggenhofer E, V Benseler, A Kroemer, FC Popp, EK Geissler, HJ Schlitt, CC Baan, MH Dahlke and MJ Hoogduijn. (2012). Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. Frontiers in Immunology 3:297.
18. de Witte SFH, F Luk, JM Sierra Parraga, M Gargesha, A Merino, SS Korevaar, AS Shankar, L O'Flynn, SJ Elliman, D Roy, MGH Betjes, PN Newsome, CC Baan and MJ Hoogduijn. (2018). Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. Stem Cells 36:602-615.
19. Fischer UM, MT Harting, F Jimenez, WO Monzon-Posadas, H Xue, SI Savitz, GA Laine and CS Cox, Jr. (2009). Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem cells and development 18:683-692.
20. Sun JH, GJ Teng, ZL Ma and SH Ju. (2008). In vivo monitoring of magnetically labeled mesenchymal stem cells administered intravascularly in rat acute renal failure. Swiss Med Wkly 138:404-12.
21. Togel F, Y Yang, P Zhang, Z Hu and C Westenfelder. (2008). Bioluminescence imaging to monitor the in vivo distribution of administered mesenchymal stem cells in acute kidney injury. Am J Physiol Renal Physiol 295:F315-21.

22. Zonta S, M De Martino, G Bedino, G Piotti, T Rampino, M Gregorini, F Frassoni, A Dal Canton, P Dionigi and M Alessiani. (2010). Which Is the Most Suitable and Effective Route of Administration for Mesenchymal Stem Cell-Based Immunomodulation Therapy in Experimental Kidney Transplantation: Endovenous or Arterial? Transplantation Proceedings 42:1336-1340.
23. Franchi F, KM Peterson, R Xu, B Miller, PJ Psaltis, PC Harris, LO Lerman and M Rodriguez-Porcel. (2015). Mesenchymal Stromal Cells Improve Renovascular Function in Polycystic Kidney Disease. Cell Transplant 24:1687-98.
24. Kunter U, S Rong, Z Djuric, P Boor, G Muller-Newen, D Yu and J Floege. (2006). Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. J Am Soc Nephrol 17:2202-12.
25. Behr L, M Hekmati, G Fromont, N Borenstein, LH Noel, M Lelievre-Pegorier and K Laborde. (2007). Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 107:p65-76.
26. Eirin A, XY Zhu, JD Krier, H Tang, KL Jordan, JP Grande, A Lerman, SC Textor and LO Lerman. (2012). Adipose tissue-derived mesenchymal stem cells improve revascularization outcomes to restore renal function in swine atherosclerotic renal artery stenosis. Stem Cells 30:1030-41.
27. Moghadasali R, M Azarnia, M Hajinasrollah, H Arghani, SM Nassiri, M Molazem, A Vosough, S Mohitmafi, M Najarasl, Z Ajdari, RS Yazdi, M Bagheri, H Ghanaati, B Rafiei, Y Gheisari, H Baharvand and N Aghdami. (2014). Intra-renal arterial injection of autologous bone marrow mesenchymal stromal cells ameliorates cisplatin-induced acute kidney injury in a rhesus Macaque mulatta monkey model. Cytotherapy 16:734-49.
28. Lee KW, TM Kim, KS Kim, S Lee, J Cho, JB Park, GY Kwon and SJ Kim. (2018). Renal Ischemia-Reperfusion Injury in a Diabetic Monkey Model and Therapeutic Testing of Human Bone Marrow-Derived Mesenchymal Stem Cells. J Diabetes Res 2018:5182606.
29. Saad A, AB Dietz, SMS Herrmann, LJ Hickson, JF Glockner, MA McKusick, S Misra, H Bjarnason, AS Armstrong, DA Gastineau, LO Lerman and SC Textor. (2017). Autologous Mesenchymal Stem Cells Increase Cortical Perfusion in Renovascular Disease. J Am Soc Nephrol 28:2777-2785.
30. Luk F, SF de Witte, SS Korevaar, M Roemeling-van Rhijn, M Franquesa, T Strini, S van den Engel, M Gargesha, D Roy, FJ Dor, EM Horwitz, RW de Bruin, MG Betjes, CC Baan and MJ Hoogduijn. (2016). Inactivated Mesenchymal Stem Cells Maintain Immunomodulatory Capacity. Stem Cells Dev 25:1342-54.
31. Gruessner RW, BK Levay-Young, RE Nakhleh, JD Shearer, M Dunning, CM Nelson and AC Gruessner. (2004). Portal donor-specific blood transfusion and mycophenolate mofetil allow steroid avoidance and tacrolimus dose reduction with sustained levels of chimerism in a pig model of intestinal transplantation. Transplantation 77:1500-6.
32. Cai J, X Yu, R Xu, Y Fang, X Qian, S Liu, J Teng and X Ding. (2014). Maximum efficacy of mesenchymal stem cells in rat model of renal ischemia-reperfusion injury: renal artery administration with optimal numbers. PLoS One 9:e92347.
33. Behr L, M Hekmati, A Lucchini, K Houcinet, AM Faussat, N Borenstein, LH Noel, M Lelievre-Pegorier and K Laborde. (2009). Evaluation of the effect of autologous mesenchymal stem cell injection in a large-animal model of bilateral kidney ischaemia reperfusion injury. Cell Prolif 42:284-97.
34. Redenbach DM, D English and JC Hogg. (1997). The nature of leukocyte shape changes in the pulmonary capillaries. Am J Physiol 273:L733-40.
35. Li J, G Lykotrafitis, M Dao and S Suresh. (2007). Cytoskeletal dynamics of human erythrocyte. Proc Natl Acad Sci U S A 104:4937-42.
36. Ekpenyong AE, N Toepfner, C Fiddler, M Herbig, W Li, G Cojoc, C Summers, J Guck and ER Chilvers. (2017). Mechanical deformation induces depolarization of neutrophils. Sci Adv 3:e1602536.

37. Fraser JK, I Wulur, Z Alfonso and MH Hedrick. (2006). Fat tissue: an underappreciated source of stem cells for biotechnology. Trends Biotechnol 24:150-4.
38. Beltrami AP, L Barlucchi, D Torella, M Baker, F Limana, S Chimenti, H Kasahara, M Rota, E Musso, K Urbanek, A Leri, J Kajstura, B Nadal-Ginard and P Anversa. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763-76.
39. Jang MJ, D You, JY Park, K Kim, J Aum, C Lee, G Song, HC Shin, N Suh, YM Kim and CS Kim. (2018). Hypoxic Preconditioned Mesenchymal Stromal Cell Therapy in a Rat Model of Renal Ischemia-reperfusion Injury: Development of Optimal Protocol to Potentiate Therapeutic Efficacy. Int J Stem Cells 11:157-167.
40. Eggenhofer E, F Luk, MH Dahlke and MJ Hoogduijn. (2014). The life and fate of mesenchymal stem cells. Front Immunol 5:148.
41. Ruster B, S Gottig, RJ Ludwig, R Bistrian, S Muller, E Seifried, J Gille and R Henschler. (2006). Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood 108:3938-44.
42. Segers VF, I Van Riet, LJ Andries, K Lemmens, MJ Demolder, AJ De Becker, MM Kockx and GW De Keulenaer. (2006). Mesenchymal stem cell adhesion to cardiac microvascular endothelium: activators and mechanisms. Am J Physiol Heart Circ Physiol 290:H1370-7.
43. Brasile L, N Henry, G Orlando and B Stubenitsky. (2019). Potentiating Renal Regeneration Using Mesenchymal Stem Cells. Transplantation 103:307-313.
44. Kim HK, SG Lee, SW Lee, BJ Oh, JH Kim, JA Kim, G Lee, JD Jang and YA Joe. (2019). A Subset of Paracrine Factors as Efficient Biomarkers for Predicting Vascular Regenerative Efficacy of Mesenchymal Stromal/Stem Cells. Stem Cells 37:77-88.