The Phenotypic Fate of Bone Marrow-Derived Stem Cells in Acute Kidney Injury

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Key Words
Acute kidney injury • Bone marrow transplantation • Granulocyte colony stimulating factor (G-CSF) • Renal stem cells

Abstract

\textbf{Background:} Despite increasing attention on the role of bone marrow derived stem cells in repair or rejuvenation of tissues and organs, cellular mechanisms of such cell-based therapy remain poorly understood. \textbf{Methods:} We reconstituted hematopoiesis in recipient C57BL/6J mice by transplanting syngeneic GFP\textsuperscript{+} bone marrow (BM) cells. Subsequently, the recipients received subcutaneous injection of granulocyte-colony stimulating factor (G-CSF) and were subjected to acute renal ischemic injury. Flow cytometry and immunostaining were performed at various time points to assess engraftment and phenotype of BM derived stem cells. \textbf{Results:} Administration of G-CSF increased the release of BM derived stem cells into circulation and enhanced the ensuing recruitment of BM derived stem cells into injured kidney. During the second month post injury, migrated BM derived stem cells lost hematopoietic phenotype (CD45) but maintained the expression of other markers (Sca-1, CD133 and CD44), suggesting their potential of transdifferentiation into renal stem cells. Moreover, G-CSF treatment enhanced the phenotypic conversion. \textbf{Conclusion:} Our work depicted a time-course dependent transition of phenotypic characteristics of BM derived stem cells, demonstrated the existence of BM derived stem cells in damaged kidney and revealed the effects of G-CSF on cell transdifferentiation.

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Introduction

The discovery of developmental plasticity of bone marrow (BM) derived stem cells has led to rapidly growing interest in their therapeutic potential in various debilitating diseases [1]. The understanding on the role of BM stem cells in the regeneration of renal tissue has evolved considerably in the last decade. Pioneering work using cell tracking (e.g. GFP, LacZ) or gender mismatching (FISH analysis) techniques showed that intrinsic regenerative process following acute kidney injury (AKI) should be ascribed to the mobilization and repopulation of BM stem cells [2-5]. Subsequently, a variety of studies suggested that endogenously mobilized [6, 7] or exogenously administered [8-11] BM stem cells could respond to the ischemic/toxic insult, home to site of renal injury and contribute significantly to the kidney regeneration. By contrast, many experiments focusing on the importance of resident renal (stem) cells revealed that cells of renal, rather than bone marrow origin, predominantly participate in tubular regeneration following AKI [12-17].

Although it is now generally recognized that BM stem cells have a limited ability to become renal tubular epithelial cells, this cell population still holds great promise in supplying novel therapeutic strategies for kidney disease. BM stem cells exert renoprotective effects by means of differentiating into or fusing with tubular cells, resulting in adopting epithelial phenotype and replenishing regenerated cells [3, 18]. Besides, there are intriguing indications that BM stem cells provide paracrine growth factor support (i.e. delivery of small molecules such as cytokines to the injured site) [19-23]. Thus, further studies are needed to address the exact cell mechanisms underlying their beneficial intervention [24].

There are possibilities for accelerating kidney repair from extra-renal cells with therapeutic interventions. Granulocyte-colony stimulating factor (G-CSF) administration can effectively mobilize BM stem cells for therapeutic purpose in clinical settings [25] and experimental models [26]. BM stem cells mobilization with the treatment by G-CSF in mice suffering AKI has been shown to enhance the recruitment of BM stem cells into the affected regions and modulate renal repair [6, 7]. Thus, understanding the cellular events and cytokines involved in regeneration of renal cells is indispensable for developing cell-based therapeutics. The present study is designed to examine whether BM stem cells undergo phenotypic transition with a time course-dependent pattern following engraftment into ischemically injured kidney. Moreover, the effects of G-CSF on migration of BM stem cells as well as on transdifferentiation of BM stem cells into bone marrow-derived renal stem cells (BMRSCs) were also evaluated.

Materials and Methods

Bone marrow transplantation (BMT) and hematopoietic reconstitution

Animal studies were in accordance with the guidelines of Nankai University Animal Care and Use Committee. Eight to 10-week-old female recipient mice (C57BL/6J) were purchased from The Laboratory Animal Center of The Academy of Military Medical Sciences. Mice were irradiated with 9.5-Gy of whole-body γ-irradiation in 2 divided doses, 2 hours apart. For bone marrow transplantation, wild-type recipient mice were injected with 0.2 ml PBS with 2.0 × 10^5 BM mononuclear cells from male β-actin-EGFP transgenic C57BL/6J mice via the retro-orbital sinus within 2 hours after irradiation. Five weeks later, BMT efficiency in recipients was determined by analyzing GFP expression of cells in peripheral blood. After the hematopoietic reconstitution, recipients were randomly assigned either to the saline group or G-CSF group (n = 20 per group, Fig. 1), followed by unilateral renal ischemic/reperfusion (I/R) injury. To evaluate the phenotypic transition of BM stem cells and the effect of G-CSF, mice were sacrificed at different time points (1, 2, 4, 8 weeks after injury, n = 3 at each time point) for histological and flow cytometric analysis.

Mobilization of BM stem cells following ischemic injury

For BM stem cells mobilization, mice were injected subcutaneously with G-CSF (recombinant human granulocyte-colony stimulating factor, Huabei Pharmaceutical Co. Ltd., Hebei, China) 200 μg/
kg daily for 9 days from day 5 before induction of ischemia. Control mice received an injection of saline (n = 20 per group).

Flow cytometry analysis

To evaluate stem cell mobilization, flow cytometry (FCM) analyses (FACScan flow cytometer; Becton Dickinson) were performed following G-CSF or saline administration. Mice were anesthetized with chloralhydrate, and peripheral blood was obtained via the retro-orbital plexus using a heparinized capillary pipette (Fisher Scientific Co., USA). Red blood cells were lysed using Lysing Buffer (Sigma). The remaining cells were stained with APC-labeled rat anti-mouse CD34 and CD45, PE-labeled rat anti-mouse CD90, CD133, and Flk-1 (all from BD Pharmingen). Five weeks after BMT, hematopoietic reconstitution in recipients was determined by analyzing GFP expression of peripheral blood.

Mouse model of renal ischemia/reperfusion injury

Five weeks after BMT, acute unilateral kidney I/R injury was carried out following the confirmation of hematopoietic reconstitution in recipient mice. Mice were anesthetized with 320mg/kg chloralhydrate injected intraperitoneally. Animals were placed on a heating pad to maintain a constant temperature (37°C). Following a midline abdominal incision, left kidney was exposed and the renal pedicle was clamped with a nontraumatic clamp for 30 minutes, meanwhile the abdomen was covered with gauze moistened in saline. Subsequently, the clamp was removed and reperfusion was confirmed visually, followed by wound suture. Then the animals were allowed to recover, with free access to food and water. Sham-operated mice were subjected to the same surgical procedure of I/R without clamping the renal vessels.

Isolation and characterization of engrafted BM stem cells after BMT

At various time points post I/R injury (1, 2, 4, and 8 weeks), mice were euthanized and the kidneys were thoroughly perfused with saline to remove blood from the vascular beds. One portion of kidney tissue was minced and added to 10 ml of a 4 mg/ml solution of dispase (sigma-Aldrich) in DMEM (Invitrogen). The minced tissue and media were transferred to a 50-ml Erlenmeyer flask and incubated for 1h at 37°C. Following the incubation, the tissue was filtered through a 40 μm nylon cell strainer (BD Pharmingen) to remove cell segments. Kidney cells suspension was washed twice in DMEM prior to FACS analysis. The renal cell samples were either directly evaluated for the expression of GFP or stained with APC-labeled rat anti-mouse CD45, PE-labeled rat anti-mouse Sca-1, CD44, and CD133 (all from BD Pharmingen).

Histology and immunohistochemistry

At various time points (2, 4, and 8 weeks), mice were euthanized and the kidneys were perfused. The specimens of kidney were embedded into paraffin or OCT compound (Miles Scientific), then sectioned to 5 μm slides and processed for immunostaining. To track BM-derived cells in kidneys, monoclonal anti-mouse Sca-1 (Cedarlane) and rat anti-mouse CD45 (BD Pharmingen) were used. Alexa Fluor 594 and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) were applied appropriately. DAPI was used for nuclear counterstaining. The numbers of positively stained cells were counted by a blinded investigator in 12 randomly selected high-power fields (magnification × 400) using a fluorescence microscope.
Statistical analysis

All data are presented as means ± SD. One-way analysis of variance was employed for comparing differences between groups. Least significant difference (equal variances) and Dunnett’s T3 (non-equal variances) post hoc tests were used for testing the differences between groups. All tests were two-tailed, and a p-value of < 0.05 was considered significant.

Results

Hematopoietic reconstitution in recipient mice

To determine whether transplantation of donor derived cells reconstituted hematopoiesis in recipient mice after lethal irradiation, the percentage of GFP+ cells in peripheral blood was evaluated by FCM. Robust expression of GFP (94.72 ± 4.16%) in chimeric mice (n = 40) was detected (Fig. 2C), indicating a complete hematopoietic reconstitution after BMT.

G-CSF administration increased the frequency of circulating BM stem cells

To assess whether administration of G-CSF could mobilize BM stem cells into peripheral blood, the expression of several stem cell markers (CD34, CD90, CD133, and Flk-1) were examined by FCM (Fig. 2A). The percentages of stem cell markers were significantly higher...
in G-CSF group compared with Saline group (1.53 ± 0.37% vs. 0.56 ± 0.18% for CD34, 7.35 ± 1.64% vs. 2.48 ± 0.53% for CD90, 5.47 ± 1.36% vs. 2.26 ± 0.58% for CD133, and 4.02 ± 0.94% vs. 1.86 ± 0.39% for Flk-1, respectively; n = 10, p < 0.0001) (Fig. 2B). These results indicate that G-CSF treatment induced significant BM stem cells mobilization from bone marrow into circulation.

Migration of BM cells into damaged kidney in a time-dependent manner and can be enhanced by G-CSF

We next investigated whether BM cells can migrate to ischemically injured kidneys. Four weeks after injury, kidneys from both groups were harvested to assess cell engraftment by direct immunofluorescent detection. Damaged kidneys from wild-type mice that underwent no irradiation or BMT were regarded as negative control. Figure 3A showed more GFP+ cells migrated to the injured kidneys in comparison to the uninjured ones at 4 weeks. Of note, there were more GFP+ cells infiltrated in kidneys from G-CSF-treated mice than in saline-treated mice. Moreover, FCM analysis of digested kidney demonstrated that no significant difference of GFP+ cells engraftment was found between two groups on week 2 (Fig. 3B). Whereas more GFP+ cells were detected in injured kidneys in G-CSF group (15.98 ± 2.52%) than that in saline group (9.50 ± 1.83%) 4 weeks post ischemic injury (p < 0.05). Moreover, the residency rate of GFP+ cells 8 weeks post injury appeared similar to that at 4-week time point.

G-CSF enhanced the engraftment of BM stem cells into kidneys following injury

To determine the identity of migrated GFP+ cells, we next performed immunofluorescent staining and FCM analysis. More GFP+/Sca-1+ cells (regarded as BM stem cells) infiltrated within injured kidneys in saline-treated mice at week 4 compared to that at week 2 (Fig. 4A),
which is due to the lower rate of GFP+ cells engraftment at 2-week time point. Note that the majority of Sca-1+ cells might represent renal resident stem/progenitor cells (GFP- cells). Four weeks after injury, administration of G-CSF nearly doubled the percentage of GFP+/Sca-1+ cells (6.75 ± 1.38%) in injured kidney compared to that in saline-treated group (3.84 ± 0.75%) (p < 0.05) (Fig. 4B and 4C). Moreover, the engraftment fashion of BM stem cells at 8-week time point resembled that at 4-week time point. Similar results were observed using other stem cell markers including CD44 and CD133 (Fig. 4D and 4E). These indicate that migration of BM stem cells into kidneys can be enhanced by G-CSF at late period post injury.
Fig. 5. The transdifferentiation of BM stem cells towards BMRSCs at late period following I/R injury. (A) Sections from saline-treated mice were stained with CD45 (red) on week 2 and 4. [scale bar = 50 μm (left) and 25 μm (right)] (B) Representative images showing recruitment of GFP+/CD45− cells (arrowhead) into kidneys 4 and 8 weeks post injury. (scale bar = 25 μm) (n = 12 random fields) (C) Quantitative evaluation of this cell population per high power field (HPF). HPF represents original magnification × 400. (D) FCM analysis of GFP+/CD45− cells in kidneys (E, F) Quantification of FCM analysis revealed a drastic decrease of CD45+/GFP− cells at 8-week time point as compared to that at 4-week time point (*p < 0.05, **p < 0.01, ***p < 0.001). Three mice in each group at different time points were examined. Abbreviation: Contra, contralateral; GFP, green fluorescent protein.
Migrated BM stem cells lost hematopoietic phenotype at late period

To investigate whether BM stem cells maintain hematopoietic lineage or undergo phenotypic transition, immunofluorescent staining and FCM analysis were performed to delineate the expression of CD45 (a hematopoietic marker) in recruited GFP+ cells post renal injury. Cells doubly positive for GFP and CD45 were detected in injured kidneys of saline-treated mice at 2 and 4 weeks (Fig. 5A). GFP+/CD45+ cells were rarely observed in injured kidneys (in both groups) 8 weeks after injury, contrasted by more such cells detected at 4-week time point (Fig. 5B). More GFP+/CD45+ cells were detected in injured kidneys in G-CSF-treated mice (13.07 ± 2.28%) than that in saline-treated ones (8.06 ± 2.06%) 4 weeks post injury (p < 0.05). Importantly and surprisingly, there was a sharp decrease in the percentage of GFP+/CD45+ cells in damaged kidneys in both groups at 8-week time point (Fig. 5D and 5E). Note that such time-dependent decrease in the ratio of GFP+/CD45+ cells appeared more obvious in G-CSF group (p = 0.001) than in saline group (p = 0.0084) (Fig. 5F), indicating G-CSF enhanced the phenotypic transition of BM stem cells.

Engrafted GFP expressing cells transdifferentiated into renal cells at late period

Six months after I/R injury, the majority of GFP+ cells were distributed diffusely in the kidney, e.g. cortex, corticomedullary junction, and medulla (Fig. 6). These cells were predominantly detected in glomeruli and interstitium, while scarcely observed in renal tubule. What is note, the number of infiltrating GFP+ cells within injured kidney showed no significant difference between two groups (data not shown), suggesting BM derived stem cell transdifferentiated into renal components in long term.

Discussion

Our current work presents a time-course dependent conversion of phenotypic properties of migrated BM stem cells as well as the existence of BM derived renal stem cells.
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(BMRSCs) in kidney during late periods after I/R injury. Meanwhile, our results indicate that G-CSF treatment could significantly augment such phenotypic transition.

We first evaluated the engraftment modality of BM cells post renal injury. Our results, similar to previous work [4, 6], showed that approximately 10% and 16% of total renal cells are of donor origin in saline- and G-CSF-treated mice on week 4 after ischemia respectively. Interestingly, Broekema et al reported that tubular reepithelialization had already ongoing by the time of emerging tubular epithelial engraftment of BM cells, which possibly explained the low number of migrated BM cells [27]. The second purpose of our study was to investigate the time course of transdifferentiation of migrated BM stem cells. Since common leukocyte antigen CD45 is only expressed on the cell surface of hematopoietic cells, it can be employed to administer lineage tracing of engrafted cells. The engrafted GFP+ cells underwent a significant decrease in the expression of CD45 8 weeks after injury. This phenotype loss indicates that the majority of recruited BM stem cells may transfer into non-hematopoietic cells. Such transdifferentiation possibly represents conversion of BM stem cells toward mature renal cells in response to the local environment. Alternatively, this transition implies transdifferentiation of BM stem cells into BMRSCs, under the consideration of the finding that the number of GFP+/Sca-1+ cells at the same time point remained consistently stable. Similarly, a more detailed investigation of the phenotypic change in engrafted BM cells revealed that no CD45 positive cells were detected in the tubules 4 weeks post renal injury, indicating the hematopoiesis ability of these BM stem cells was depleted [15]. Additionally, Dekel et al. reported the existence of resident renal stem cells that express Sca-1 and lacks CD45 [28]. In this regard, engrafted BM stem cells may lose their inherent phenotypic characteristic and switch to a non-hematopoietic lineage, possibly BMRSCs at an intermediate stage.

Herein, exogenous G-CSF administration markedly increased the release of BM stem cells into circulation, thereby facilitating their homing to injured sites. Previous data showed that I/R injury can increase the serum level of G-CSF on both mRNA and protein levels [29]. This finding coupled with our data, suggests a complex interplay between exogenous/endogenous G-CSF administration and renal injury. Evidence is accumulating that G-CSF treatment with [6, 7] or without [30, 31] consequent enhanced migration of BM stem cells can attenuate renal tubular injuries in mouse models induced by toxic and ischemic insults. In this study, the number of GFP+/Sca-1+ cells within injured kidney in G-CSF-treated mice was 1.8 times higher than that in saline-treated mice, suggesting the effectiveness of hematopoietic cytokine in BM stem cells recruitment. Intriguingly, G-CSF can augment phenotypic transition of engrafted BM stem cells in damaged kidney in current study. Thus, the transdifferentiation of recruited BM stem cells is predominantly dependent upon the efficiency of G-CSF-induced mobilization. Although the mechanism underlying the effects of G-CSF on stem cell mobilization and transdifferentiation is not addressed herein, SDF-1 and CXCR4 interactions have been extensively investigated [32]. A more recent work showed that Sca-1+ cardiac stem cells could exert cardioprotection via SDF-1 in a murine model of myocardial I/R [33].

In conclusion, our data demonstrates that acute ischemic injury induced a phenotypic transition of BM stem cells, which can be enhanced by G-CSF administration. The ability of BM stem cells to acquire the phenotypic properties of resident renal stem cells in kidney after engraftments suggests that BM stem cells could potentially differentiate into mature cells during kidney regeneration. Thereby, strategies to enhance the transdifferentiation of BM stem cells into BMRSCs and renal cells may consolidate cell-based therapies for renal injury.

Conflict of Interests

None.
Acknowledgements

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