Objectives: We investigated the time course of the stromal cell-derived factor 1α (SDF1α) expression and behavior of intravenously administered bone marrow-derived stromal (BMS) cells in the urinary bladder of partial bladder outlet obstruction (PBOO) rats.

Methods: Study 1: Recombinant SDF1α or saline was directly injected into the bladder wall of female rats followed by intravenous administration of BMS cells isolated from green fluorescent protein (GFP) transgenic rats. The bladder was examined with immunohistochemistry to determine whether SDF1α would enhance migration of BMS cells to the bladder. Study 2: Following surgery of PBOO or sham in female rats, bladders were removed on days 1–14, and expression of hypoxia inducible factor 1α (HIF1α) and SDF1α were examined with real-time polymerase chain reaction (PCR) to determine if PBOO preferentially increased their expression. Study 3: Female rats underwent PBOO or sham surgery followed by intravenous administration of GFP-positive BMS cells. Bladders were examined with immunohistochemistry on days 1–14 to determine whether BMS cells preferentially accumulated in the bladder.

Results: BMS cells were accumulated in the injection site of SDF1α but not saline in the bladder. SDF1α and HIF1α increased at day 1 after PBOO compared to sham. More BMS cells accumulated in the bladder of PBOO on day 1, and some BMS cells expressed smooth muscle phenotypes by day 14.

Conclusion: SDF1α induced with ischemia/hypoxia due to PBOO is implicated in the accumulation of BMS cells in the bladder and regeneration of the bladder for PBOO.

Key words bladder outlet obstruction, bone marrow-derived stromal cell, hypoxia inducible factor 1α, regeneration, stromal cell-derived factor 1α

1. INTRODUCTION

Attempts to promote regeneration of the urinary tract in animal models using injections of cultured bladder smooth muscle and urothelial cells seeded onto bioengineered scaffolds have had limited success. As some problems, such as insufficient graft survival and the shrinkage of the graft, currently occur in basic research, these maneuvers are not yet suitable for clinical application.1–4 The recent new technology of tissue engineering and cell therapy as an alternate repair strategy is focused on practical use to restore function of injured organs and tissues using mesenchymal stem cells. The existing structure of the organ works to maintain the microenvironment that is necessary for cell survival and sustenance. It is recognized that several types of cells, such as smooth muscle cells and neural cells, are differentiated from pluripotent stem cells in the injured organs, and the injured organs are repaired and their function is restored by cell therapy using pluripotent stem cells.5 In fact, the feasibility of cell therapy using mesenchymal stem cells, which are a type of pluripotent stem cell, has been reported in many animal models, such as cardial infarction model,6 and spinal cord injury model.8 A clinical trial for humans has already been performed in patients with cardiac infarction.9 However, very little research has been performed investigating the recruitment of mesenchymal stem cells into an injured bladder model,10,11 and cell therapy using mesenchymal stem cells has not been established in the urological field.

For future progress of tissue engineering, it is necessary to clarify the mechanism of administered cells as cell therapy, which migrate and accumulate in the injured organs and tissues. Focused on chemokine, an hypoxic environment in bladder tissues, which induces expression of hypoxia inducible factor 1α (HIF1α), in turn stimulates expression of a chemokine, stromal cell-derived factor 1α,
factor 1α (SDF1α [CXCL12]). SDF1α binds to the CXCR4 receptor expressed on mesenchymal stem cells. SDF1α controls the migration and accumulation of mesenchymal stem cells in ischemic tissues, which has the potential to repair injured tissues. Although it is speculated that a similar mechanism would work in the bladder, the investigation of the SDF1α expression in bladder tissue has not been done yet and changes of SDF1α expression in injured bladder tissue remain unknown. Moreover, it has not been revealed how mesenchymal stem cells play a role in the remodeling process of injured bladder tissue.

In the present study, we investigated (i) whether injection of SDF1α in a non-injured bladder would induce migration of intravenously administered bone marrow-derived stem (BMS) cells to the bladder, (ii) whether SDF1α and HIF1α expression are increased in the partial bladder outlet obstruction (PBOO) bladder, and (iii) whether intravenously administered BMS cells would migrate to the PBOO bladder.

2. METHODS

2.1. Laboratory animals

Wild-type 10-week-old female Sprague–Dawley (SD) rats were purchased from Japan Slc Inc (Shizuoka, Japan). Enhanced Green Fluorescent Protein (EGFP) transgenic SD rat, generated by Dr Okabe (Osaka University, Osaka, Japan), were purchased from Japan Slc. Inc. Animals were treated in accordance with NIH animal care guidelines and all animal experiments were approved by the Hokkaido University Animal Experiment Committee.

2.2. Isolation and culture of BMS cells

Bone marrow was harvested from tibia and femur dissected from EGFP transgenic rats, euthanized by diethyl ether overdose. Bone marrow was flushed off from the bones with Dulbecco’s modified Eagle medium (DMEM)/Ham’s F12 (1:1) (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) (GIBCO, NY, USA). The flushed bone marrow was pipetted, filtered through 150-μM steel mesh and incubated in DMEM/Ham’s F12 containing 10% FBS under standard culture condition. Seventy-two hours later, and on alternate days thereafter, the culture medium was replaced. Adherent cells were cultured until confluent and subjected to subsequent experiments.

2.3. Procedures for PBOO

Female SD rats were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg). The bladder was exposed through a lower abdominal incision. Recombinant murine SDF1α (8 μg/kg; Peprotech, London, UK) diluted in a total volume of 60 μL saline (SDF1α group) or 60 μL saline (vehicle group) was administered into the several regions of the bladder by direct bladder wall injection with the microsyringe in the SDF1α group (n = 3) or the vehicle group (n = 3). At the same time, they were administered BMS cell (3 × 10⁷) from EGFP transgenic rats through the tail vein. After 12 h, the rats were anesthetized with pentobarbital, and perfusion fixed with 4% PFA. The bladders were dissected and examined with immunohistochemistry.

To create infravesical obstruction, a metal rod of 0.45 mm in diameter was placed beside the proximal urethra and a 4-0 silk ligature was tied around the urethra and metal rod. Subsequently, the metal rod was removed and the abdominal incision was closed. Control rats underwent sham surgery in which the urethra was circumferentially dissected, but not ligated, and the abdominal incision was closed.

2.4. Immunofluorescence microscopy

Animals were anesthetized with intraperitoneal injections of sodium pentobarbital (100 mg/kg) and killed by intracardiac perfusion with 200 mL phosphate buffer (PB) followed by 500 mL ice cold 4% paraformaldehyde (PFA) fixative in PB. The bladder was then removed and post-fixed for 24 h in the same fixative at 4 °C followed by cryoprotection in phosphate-buffered 30% sucrose solution for 3–5 days. Tissues were serially blocked, embedded in optimal cutting temperature (OCT) compound (Sakura Finetechical Co., Tokyo, Japan) and kept at −80 °C. Frozen sections of 10 μm thickness were cut with microtome and the sections were attached to positively-charged slides. For immunofluorescence histochemistry, the sections were washed with phosphate buffer saline (PBS), and permeabilized with 0.3% Triton-X100 for 5 min. The sections were blocked with 10% normal goat serum (Vector Laboratories, CA, USA) for 60 min at room temperature. Sections were treated overnight at 4 °C with a rabbit polyclonal antibody (1 : 200, anti-GFP antibody; CHEMICON international Inc., CA, USA) and a mouse monoclonal antibody (1:400, anti-alpha smooth muscle actin [SMA] antibody; Sigma). Samples were washed with PBS and treated with fluorescein conjugated anti-rabbit IgG antibodies (1:200; Jackson Immunoresearch, PA, USA) and rhodamine conjugated anti-mouse IgG antibodies (1:200; Jackson ImmunoResearch, PA, USA). The slides were washed with PBS and mounted with Permafluor (Beckman Coulter, CA, USA). The sections were observed under confocal laser fluorescence microscope (MRC-1024; Bio-Rad Laboratories, CA, USA).

2.5. Study 1: Assessment of BMS cells accumulating in the normal bladder wall followed by injection of recombinant SDF1α protein

Wild-type female SD rats were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg). The bladder was exposed through a lower abdominal incision. Recombinant murine SDF1α (8 μg/kg; Peprotech, London, UK) diluted in a total volume of 60 μL saline (SDF1α group) or 60 μL saline (vehicle group) was administered into the several regions of the bladder by direct bladder wall injection with the microsyringe in the SDF1α group (n = 3) or the vehicle group (n = 3). At the same time, they were administered BMS cell (3 × 10⁷) from EGFP transgenic rats through the tail vein. After 12 h, the rats were anesthetized with pentobarbital, and perfusion fixed with 4% PFA. The bladders were dissected and examined with immunohistochemistry.
2.6. Study 2: mRNA expression of HIF1α and SDF1α in the PBOO bladder

In the PBOO group, female SD rats underwent PBOO surgery. On postoperative days 1, 3, 7, 10 and 14 (n = 6 in each), rats were euthanized by pentobarbital overdose (100 mg/kg) and the bladders were exposed. In the sham group, female SD rats underwent sham surgery. On postoperative day 1 (n = 6), rats were euthanized by pentobarbital overdose (100 mg/kg) and the bladders were exposed. After the bladder weight was measured, the bladders were immediately frozen with liquid nitrogen, and preserved at −80 °C until use. The total RNA was isolated from the bladder tissues using TRI REAGENT (Sigma, MO, USA). The first strand cDNA synthesis was performed on 3 μg of total RNA using the Moloney Murine Leukemia Virus (MMLV) converting enzyme (Invitrogen, CA, USA). PCR product was detected as an increase in fluorescence with ABI Sequence Detection System (Applied Biosystems, CA, USA). PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) and RT products. As the amplification primers and probes, we used TaqMan Gene Expression Assays (Applied Biosystems) primers and TaqMan probes. Relative quantification PCR products were calculated after normalization to Ribosomal 18S rRNA (rS18). The gene expression of each group was shown relatively by estimating the gene expression of sham group as 1.

2.7. Study 3: Assessment of BMS cells accumulating in the PBOO bladder

Female SD rats underwent PBOO or sham surgery, followed by intravenous administration through the tail vein of BMS cells (5 x 10⁶) from EGFP transgenic rats. The PBOO group was anesthetized with pentobarbital (100 mg/kg), and perfusion fixed with 4% PFA. The bladders were dissected and examined with immunohistochemistry on postoperative day 1 (n = 3) and day 14 (n = 3). In the sham group, the bladders were dissected and examined with immunohistochemistry on postoperative day 1 (n = 3).

2.8. Statistical evaluation (data analysis)

Results are expressed as mean ± standard error. Mann–Whitney U-test was used to perform statistical data analysis. Statistical significance was set at P < 0.05.

3. RESULTS

3.1. Study 1: Assessment of BMS cells accumulating in the normal bladder wall followed by injection of recombinant SDF1α protein

No GFP-positive BMS cells were observed in the bladder wall of animals in the vehicle group (Fig. 1a). GFP-positive BMS cells did accumulate at the site of injection in the bladder of the SDF1α group (Fig. 1b). Bladder smooth muscle was densely stained with red anti-SMA antibody in both vehicle (Fig. 1c,e) and SDF-1 (Fig. 1d,f) injected bladders.

We also examined BMS cells accumulating in the other organs besides the bladder, which revealed that BMS cells were observed in the liver, kidney, intestine, or lung (data not shown).

3.2. Study 2: mRNA expression of HIF1α and SDF1α in the PBOO bladder

3.2.1. Time course of bladder weight after PBOO surgery

Bladder weight in the sham group was 170.9 ± 23.0 mg. Bladder weight in the PBOO group increased significantly by day 7 (p < 0.05) and reached 691.8 ± 90.7 mg by day 14 (p < 0.01) (Fig. 2).

3.2.2. Time course of HIF1α-mRNA and SDF1α-mRNA expression in the PBOO bladder

Figures 3 and 4 show that HIF1α-mRNA and SDF1α-mRNA expression increased in the PBOO bladder relative to the mRNA expression in the sham bladder as 1. HIF1α-mRNA increased 7.2 times at day 1 (P < 0.05, compared to sham) in the PBOO group compared to the sham group, and then returned to sham levels after day 3 (Fig. 3).

SDF1α-mRNA increased 4.0 times, also peaked at day 1 (P < 0.05, compared to sham) after PBOO, and then returned to sham levels after day 3. However, SDF1α-mRNA gradually increased again and reached to 2.5 times on day 14 (Fig. 4).

3.2.3. Study 3: Assessment of BMS cells accumulating in the PBOO bladder

No GFP labeled BMS cells were found in the bladder in the sham group (Fig. 5a–c). In the PBOO group BMS cells were present and most of the BMS cells were distributed in the interstitial tissue below the bladder mucosa and the serosa on day 1 (Fig. 5d–f). BMS cells were also migrating into the interstitial tissue of the muscle layer (Fig. 5d–f). No GFP-positive BMS cells were present in the PBOO bladder on day 1 stained with SMA in the muscle layer (Fig. 5d–f) and thus showed no sign of differentiation into a muscle phenotype. Many BMS cells migrated in the PBOO bladder were also observed on day 14 and GFP-positive cells were migrated between the smooth muscle layer (Fig. 5g–i). Some GFP-positive cells (approximately less than 10%) on the smooth muscle layer showed fusiform morphologically and stained with SMA, suggesting that BMS cells differentiated into the smooth muscle-like phenotypes in the muscle layer (Fig. 5j).

4. DISCUSSION

Lower urinary tract (LUT) obstructive diseases, including benign prostate hyperplasia, result in voiding and storage dysfunction, associated with pathological changes in bladder tissue. Investigation to clarify the mechanism of these LUT obstructive diseases is possible to link to the invention of the newer therapeutic concepts for voiding and storage dysfunction. In the present study, we focused on the expression of the chemokine SDF1α...
and the migration of intravenously injected BMS cells in PBOO bladder tissue. We show that increases in exogenously administered SDF1α protein are associated with BMS cells accumulating in the normal bladder in vivo, that SDF1α-mRNA level is increased in the PBOO bladder, and that BMS cells injected intravenously accumulate in the PBOO bladder and that some BMS cells differentiate into smooth muscle phenotypes. This approach suggests a strategy for restoration of bladder muscle.
Chemokines are a large family of structurally homologous cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from the blood to the tissues. Chemokines are classified into four subfamilies of chemokines (CXC, CC, C, CX3C). SDF1α is a member of CXC and is the only known ligand for the chemokine receptor CXCR4. Absence of SDF1α or the CXCR4 receptor is embryologically lethal; SDF1α and the CXCR4 receptor regulate embryonic development, including hematopoiesis, cardiogenesis, chemotaxis, cerebellar development, vascularization of gastrointestinal tract, and accumulation of myeloid stem cells form the fetal liver to the bone marrow environment. Tissue hypoxia is thought to induce expression of transcription factor HIF1α, which stimulates SDF1α gene expression; SDF1α expression increases adhesion, migration and accumulation of circulating BMS cells in ischemic tissues through CXCR4-interaction.

Bladder weight is rapidly increased in the PBOO bladder due to hypertrophy of bladder smooth muscle cells, uroepithelial cells and fibroblast cells; our results confirmed a significant increase bladder weight in the PBOO group by day 7. This may contribute to ischemia in the PBOO bladder, which is likely to be an important factor contributing to bladder pathology.

It has been reported that PBOO makes bladder tissue ischemic and that HIF1α protein expression increases in the PBOO bladder and is associated with processes after ischemia such as erythropoiesis, angiogenesis, cell growth, differentiation, survival or apoptosis. HIF1α-mRNA increased at day 1 after PBOO surgery and then decreased, suggesting a temporary ischemia, because the half-life of HIF1α is short. We speculated that some sort of compensation for ischemia could occur after PBOO and HIF1α was normalized within 3 days after PBOO. However, as HIF1α usually reflects acute ischemia, not chronic ischemia, chronic ischemia could continue even after normalization of HIF1α. SDF1α increased on day 1 after PBOO. In accord with the paper by Ceradini et al., these phenomena could be considered that PBOO made bladder tissue ischemic and induced SDF1α-mRNA via HIF1α. Interestingly, SDF1α-mRNA increased again after the nadir on day 3. This re-increase of SDF1α-mRNA did not link to the HIF1α-mRNA change. The alternative pathway except for the HIF1α-SDF1α interaction could contribute to this mechanism, as nitric oxide (NO) and NF-κB were reported to induce SDF1α expression. Further studies are necessary to clarify this mechanism of increased SDF1α.

With regard to SDF1α and BMS cells accumulating in the bladder tissues, we have also shown that BMS cells can migrate to the bladder tissue even in the normal bladder by injection of SDF1α recombinant protein. In the other organs, similar phenomena have been reported previously. From this study, SDF1α injected into the bladder is thought to make circulating BMS cells accumulate in the bladder tissue. This result means that SDF1α can be an essential factor for accumulating of BMS cells.
in the organ. In the PBOO bladder, BMS cells, which were administered intravenously at the time of the PBOO surgery, were observed on day 1 as well as day 14 in the present study. As SDF1α-mRNA was increased in the bladder tissue after PBOO surgery, it is speculated that those BMS cells migrated and accumulated in the bladder with the attraction of SDF1α stimulated by PBOO.

Mesenchymal stem cells can differentiate into the various mesodermic organs. BMS cells can differentiate into myocardial cells and cell therapy using BMS cells has a potential to be a new therapeutic modality. Here myocardial cells and cell therapy using BMS cells has aious mesodermic organs. BMS cells can differentiate into the ischemic myocardial tissues increased the number of BMS cells in the heart and improved cardiac function within 14 days, which could be one of the reasons why BMS cells were not differentiated into the other tissues except smooth muscle. This study was observed within 14 days, which could be one of the reasons why BMS cells were not differentiated into the other tissues except smooth muscle. BMS cells are also amenable to genetic manipulation to produce bioactive molecules, which could further enhance the therapeutic value of non-invasive intravenous administration.

We have not examined how the function of the PBOO bladder would be changed by BMS cells nor have we evaluated the extent of integration of these cells into the bladder tissue. A similar strategy of injection of SDF1α into the ischemic myocardial tissues increased the number of BMS cells in the heart and improved cardiac function of the heart. In contrast, however, BMS cells accumulated in arteries worsened atherosclerosis. It is necessary to clarify how BMS cells migrated into the bladder affect bladder function of LUT obstructive diseases. In the future, controls of BMS cells migrated into the bladder using the SDF1α or CXCR4 mechanism would have the potential to develop the newer therapeutic modalities for the disorder of LUT obstructive diseases.

In conclusion, SDF1α induced with ischemia/hypoxia due to PBOO is implicated in the accumulation of BMS cells in the bladder and regeneration of the bladder for PBOO.

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Disclosure

There are no financial or commercial interests concerned for the authors of the present paper.


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