# 博士学位論文

## 雌性マウスにおける糖尿病による骨修復遅延におけるマクロファージとプラスミノーゲンアクチベーターインヒビター１の役割

近畿大学大学院
医学研究科医学系専攻
**下出孟史**

Doctoral Dissertation

# Role of macrophages and plasminogen activator inhibitor-1 in delayed bone repair in diabetic female mice

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論文題目　Role of macrophages and plasminogen activator inhibitor-1 in delayed bone repair in diabetic female mice

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# Role of macrophages and plasminogen activator inhibitor-1 in delayed bone repair in diabetic female mice

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Abstract

Delayed fracture healing is a significant clinical problem among various complications in diabetic patients. However, the details in the mechanisms of diabetic delayed bone repair have still remained unknown. Here, we investigated the roles of macrophages in diabetic delayed bone repair after femoral bone injury using streptozotocin (STZ)-treated or plasminogen activator inhibitor-1 (PAI-1)-deficient female mice. STZ treatment significantly decreased the numbers of F4/80-positive cells (macrophages), but not Gr-1-positive cells (neutrophils), at the damaged site on day 2 after femoral bone injury in mice. It significantly decreased the mRNA levels of macrophage colony-stimulating factor, inducible nitric oxide synthase (iNOS), interleukin (IL)-6 and CD206 at the damaged site on day 2 after bone injury. Moreover, STZ treatment attenuated a decrease in the number of hematopoietic stem cells in bone marrow from damaged femurs induced by bone injury. On the other hand, PAI-1 deficiency significantly attenuated a decrease in the number of $F4/80^+$ cells induced by STZ treatment at the damaged site on day 2 after bone injury in mice. PAI-1 deficiency did not affect the mRNA levels of iNOS and IL-6 in F4/80- and CD11b-double positive cells from the bone marrow of the damaged femurs decreased by diabetic state in mice. PAI-1 deficiency significantly attenuated the phagocytosis of macrophages at the damaged site suppressed by diabetic state. In conclusion, we demonstrated that type 1 diabetic state decreases accumulation and phagocytosis of macrophages at the damaged site during the early bone repair process after femoral bone injury through PAI-1 in female mice.

Précis

Diabetic state attenuated the accumulation and function of macrophages at the damaged site of bone during the early stage bone repair process through plasminogen activator inhibitor-1 in female mice.

Keywords：Diabetes, Bone repair, Macrophage, PAI-1, Hematopoietic stem cell

Introduction

Diabetes is characterized by chronic hyperglycemia based on impaired insulin action and secretion. Diabetic patients suffer from various complications due to microvascular and macrovascular diseases, and it is well known that cutaneous wound injury repair is delayed in diabetic patients. Recent findings suggested that fracture risk is increased mainly because of decreased osteoblastic bone formation in patients with type 1 and type 2 diabetes [1-4]. Moreover, it has been generally recognized that the diabetic state causes delayed fracture healing [5]. However, details in the mechanisms of diabetic osteoporosis and delayed bone repair still remain unclear.

Several studies revealed that the decreased mobilization of mesenchymal stem cells and hematopoietic stem cells (HSCs), vascularization, chondrogenesis and osteogenesis are related to the mechanisms of delayed bone repair in diabetes [5-7]. We reported previously that plasminogen activator inhibitor-1 (PAI-1), an inhibitor of plasminogen activator, is involved in delayed bone repair as well as osteopenia induced by streptozotocin (STZ) in female mice, although vitamin D deficiency did not affect delayed bone repair induced by type 1 diabetes in mice [8-10].

It is well known that the bone repair process consists of three phases: inflammation, restoration and remodeling [11]. In the inflammatory phase, immune cells, such as neutrophils and macrophages, migrate into the damaged bone site. Macrophages participate in tissue repair through the engulfment of cell debris, efferocytosis of damaged cells and stimulation of vessel formation [12]. Many previous studies have indicated that macrophage dysfunction is related to impaired cutaneous wound healing in diabetes [13-16]. Macrophage peroxisome proliferator-activated receptor $\gamma$ is involved in impaired cutaneous would healing in type 2 diabetic mice [11]. Moreover, advanced glycation end products (AGEs) induce a delay in cutaneous wound healing through autophagy- and macrophage change-related mechanisms in diabetic mice [17]. These findings suggest that macrophages play important roles in the tissue repair process.

As for the relationships between macrophages and bone, recent studies indicated that a specialized resident bone macrophage population, osteomacs, plays some roles in bone formation in ossification and mineralization in mice [18-21]. We reported that macrophages that migrate to the injury sites after femoral bone injury might participate in tissue fibrinolysis-related bone repair processes in mice [22, 23]. These findings suggested that macrophages migrating to bone injury sites are important for the bone repair process after bone injury or fracture. On the other hand, cutaneous wound healing is disturbed by a decrease in neutrophils from extracellular traps-driven chronic inflammation in diabetes [24, 25]. Collison et al. reported that the number of neutrophils extruding from capillaries is decreased by an increase in AGEs [26]. Taken together, neutrophils might participate in delayed bone repair in diabetes. However, the roles of macrophages and neutrophils in delayed bone repair after bone injury in diabetes have still remained unknown.

In the present study, we investigated the roles of macrophages and neutrophils in delayed bone repair after bone injury using a type 1 diabetic female mouse model induced by STZ treatment. In our previous

study, PAI-1 deficiency blunted STZ-induced diabetic osteopenia in female mice, but not male [9]. Moreover, it blunted delayed bone repair induced by type 1 diabetic state in female mice [8]. We therefore employed female mice for the present study.

## Materials and Methods

### Materials

Anti-Gr-1 and anti-Osterix antibodies were obtained from Abcam (Cambridge, UK). Anti-F4/80 and anti-stromal cell-derived factor (SDF)-1 antibodies were purchased from AbD Serotec (Raleigh, NC, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Bone morphogenetic protein (BMP)-2 was provided by Pfizer Inc. (Groton, CT, USA).

### Animal experiments

In total, 177 female mice with a mixed C57BL/6J (81.25%) and 129/SvJ (18.75%) genetic background were analyzed. We included 46 female mice with PAI-1 gene deficiency (PAI-1 KO) and 131 corresponding wild-type (WT) mice. These mice were kindly provided by Professor D. Collen (University of Leuven, Leuven, Belgium). Diabetes was randomly induced in female WT and PAI-1 KO mice (8 weeks of age) by daily injections of STZ (50 mg/kg body weight, i.p., in saline) (Sigma, St. Louis, MO, USA), a cytotoxin of pancreatic $\beta$-cells, for 4 days [9]. Control mice were injected with saline alone. Four days after the last injection, non-fasting blood glucose levels were measured using a blood glucose meter (Glutest ace; Sanwa Kagaku Kenkyusyo, Nagoya, Japan) by using blood from the tail vein. Mice with blood glucose levels >300 mg/dl were considered as diabetic. At 3 weeks after induction of diabetes, a bone injury surgery was performed in the right femur of the mice. We maintained the animals in metabolic cages with a 12-h light/12-h dark cycle; they received food and water ad libitum. All experiments were performed in accordance with the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kindai University.

### Bone injury model

A bone injury was induced in mice, as previously described [22]. Briefly, under anesthesia with 2% isoflurane, the anterior skin over the mid-femur of the right leg was incised longitudinally for a length of 5 mm. Then, we exposed the surface of the femoral bone by splitting the muscles. Thereafter, a hole was made using a drill with a diameter of 0.9 mm. To prevent thermal necrosis of the margins, saline was irrigated continuously during drilling. The incised skin was then sutured in a sterile manner, and anesthesia was discontinued. To maintain body temperature at 37℃, a heated pad was used during surgery.

### Histological analysis

The femur was removed, fixed in 4% paraformaldehyde, demineralized in 22.5% formic acid and 340 mM

sodium citrate solution for 24 h, and embedded in paraffin. Thereafter, 4-μm thick sections were obtained. Immunostaining was performed as described previously [22]. Briefly, the sections were stained with incubated with anti-Gr-1, anti-F4/80, or anti-Osterix and anti-SDF-1 antibodies followed by incubation with an appropriate second antibody conjugated with horseradish peroxidase (Table 1). Positive signals were visualized using a tyramide signal amplification system (PerkinElmer, Waltham, MS, USA). These sections were counterstained with 4', 6-diamidino-2-phenylindole and photographed with the use of a fluorescence microscope (BZ-700, Keyence, Osaka, Japan).

Supplemental Table 1. The primers for real-time PCR

| Primer | | Sequence |
|---|---|---|
| Mouse | | |
| MCSF | Forward | 5'-GACTTCATGCCAGATTGCC-3' |
| MCSF | Reverse | 5'-GGTGGCTTTAGGGTACAGG-3' |
| MCP-1 | Forward | 5'-CCACTCACCTGCTGCTACTCA-3' |
| MCP-1 | Reverse | 5'-TGGTGATCCTCTTGTAGCTCTCC-3' |
| MIP-1α | Forward | 5'-CCTCTGTCACCTGCTCAACA-3' |
| MIP-1α | Reverse | 5'-GATGAATTGGCGTGGAATCT-3' |
| IL-4 | Forward | 5'-ACAGGAGAAGGGACGCCAT-3' |
| IL-4 | Reverse | 5'-GAAGCCCTACAGACGAGCTCA-3' |
| IL-6 | Forward | 5'-GTTCTCTGGGAAATCGTGGA-3' |
| IL-6 | Reverse | 5'-GGAAATTCGGGGTAGGAAGGA-3' |
| TNF-α | Forward | 5'-CCCAGACCCTCACACTCAGATC-3' |
| TNF-α | Reverse | 5'-GCCACTCCAGCTGCTCCTC-3' |
| IL-1β | Forward | 5'-GGTCAAAGGTTTGGAAGCAG-3' |
| IL-1β | Reverse | 5'-TGTGAAATGCCACCTTTTGA-3' |
| iNOS | Forward | 5'-TTTGCTTCCATGCTAATGCGAAAG-3' |
| iNOS | Reverse | 5'-GCTCTGTTGAGGTCTAAAGGCTCCG-3' |
| IL-10 | Forward | 5'-GCTCTTACTGACTGGCATGAG-3' |
| IL-10 | Reverse | 5'-CGCAGCTCTAGGAGCATGTG-3' |
| CD206 | Forward | 5'-TTTGGAATCAAGGGCACAGAG-3' |
| CD206 | Reverse | 5'-TGCTCCACAATCCCGAACC -3' |
| Arginase1 | Forward | 5'-CTCCAAGCCAAAGTCCTTAGAG-3' |
| Arginase1 | Reverse | 5'-AGGAGCTGTCATTAGGGACATC-3' |
| TGF-β1 | Forward | 5'-GCAACAATTCCTGGCGTTACC-3' |
| TGF-β1 | Reverse | 5'-CGCTGAATCGAAAGCCCTGTA-3' |
| SDF-1 | Forward | 5'-CTGTGCCCTTCAGATTGTTG-3' |
| SDF-1 | Reverse | 5'-TCAGCCTTCCTCGGGGGTCT-3' |
| Osterix | Forward | 5'-AGCGACCACTTGAGCAAACAT-3' |
| Osterix | Reverse | 5'-GCGGCTGATTGGCTTCTTCT-3' |
| ALP | Forward | 5'-ATCTTTGGTCTGGCTCCCATG-3' |
| ALP | Reverse | 5'-TTTCCCGTTCACCGTCCAC-3' |
| GAPDH | Forward | 5'-AGGTCGGTGTGAACGGATTTG-3' |
| GAPDH | Reverse | 5'-GGGGTCGTTGATGGCAACA-3' |
| β-actin | Forward | 5'-TACCACAGGCATTGTGATGG-3' |
| β-actin | Reverse | 5'-TTTGATGTCACGCACGATTT-3' |

MCSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; IL, interleukin; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; TGF-β1, transforming growth factor-β1; SDF-1, stromal cell-derived factor-1; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Quantitative real-time PCR

Total RNA was isolated from a 5-mm piece of femur containing the damaged site or cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The incorporation of SYBR Green into double-stranded DNA was assessed by quantitative real-time PCR using an ABI StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The primers for real-time PCR are shown in Supplemental Table 1. The mRNA levels of the target genes were normalized using $\beta$-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

Flow cytometric analysis

Bone marrow cells were obtained from mice as described previously [27]. To flush the bone marrow cells from harvested femurs from 2 mice, Hanks' balanced salt solution buffer with 2% fetal bovine serum (FBS) was used. Then, bone marrow cells were added to an equivalent volume to Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden), and were harvested by centrifugation for 15 min at 630 g at 4℃. Cells were resuspended in phosphate-buffered saline supplemented with 3% FBS. The cells were analyzed with a FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA). HSCs were identified in bone marrow cell populations using Alexa 700-conjugated anti-CD34, BV711-conjugated anti-c-kit, PE-Cy7-conjugated anti-Sca-1 antibodies and the peridinin-chlorophyll protein complex-Cy5.5-conjugated anti-lineage antibodies cocktail (BD Biosciences) (Table 1). Numbers of HSCs harvested from the bone marrow of the contralateral intact and damaged femurs on day 2 after femoral bone injury were enumerated by flow cytometry.

Isolation of F4/80- and CD11b-double positive cells from the femur

The bone marrow cells were flushed out into Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin streptomycin. The cells were labeled at 4℃ for 30 min with the optimal dilution of phycoerythrin-conjugated anti-F4/80 antibody (AbD Serotec) and peridinin-chlorophyll protein-Cy5.5-conjugated anti-CD11b antibody (BD Biosciences) (Table 1). After lysis of the erythrocytes, F4/80- and CD11b-double positive cells ($5.0 \times 10^5$) were isolated using a FACS Aria II cell sorter (BD Biosciences) and analyzed by real-time PCR, as described previously [22].

Transmission electron microscopy

Transmission electron microscopy analysis was performed as described previously [23]. Briefly, mice were perfused transcardially with physiological saline and, subsequently, with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) on day 2 after femoral bone injury. Femurs were removed, demineralized in 22.5% formic acid and 340 mM sodium citrate solution, and post-fixed in the same fixative overnight at 4℃. After fixation in 1% buffered osmium tetroxide and pre-staining with 0.5% uranyl acetate, small pieces of the femurs were embedded in epoxy resin and 70-nm thick sections were obtained from the damaged site. The ultra-thin sec-

tions were stained with 3% uranyl acetate for 20 min at room temperature. The stained sections were photographed using an electron microscope (HT-7700; Hitachi High-Technologies Co., Tokyo, Japan) at an accelerating voltage of 100 kV. Activity of macrophage phagocytosis at the damaged site of femur was quantitatively analyzed as described previously [23]. Briefly, at least 25 macrophages at the damaged site of femur were photographed in each mouse, and number of phagocytosing macrophages with erythrocytes or cellular debris longer than 2 $\mu$m in diameter was quantified in a blinded manner. Then, the ratio of phagocytosing macrophage number to subject macrophage was calculated in each mouse for the assessment of phagocytosis activity.

### Preparation of bone marrow stromal cells

Bone marrow stromal cells were obtained from female WT and PAI-1 KO mice as described previously [22]. Briefly, femurs and tibias were removed from the mice and cleaned of soft tissue. The bone marrow cells were flushed out using DMEM. After the cells were grown in DMEM with 10% FBS for 24 h, the nonadherent cells were removed using phosphate-buffered saline.

### Preparation of primary osteoblasts

Calvarial osteoblasts were obtained from WT and PAI-1 KO mice in accordance with the method as described previously [22]. Briefly, calvaria from 3-day-old female WT and PAI-1 KO mice were digested four times with minimum essential medium alpha modification ($\alpha$-MEM) containing 1 mg/ml collagenase and 0.25% trypsin for 20 min at 37℃. The cells from the second, third and fourth digestions were collected and cultured in $\alpha$-MEM with 10% FBS.

### Statistical analysis

All data are represented as means ± SEM. Statistical significances were assessed using Mann–Whitney *U* test for comparisons of two groups. Normality of distribution was tested using Shapiro–Wilk test for multiple comparisons. Two-way ANOVA followed by Tukey–Kramer test was performed when data presented a normal distribution. Dunn test was performed when data did not present a normal distribution for multiple comparisons. Differences among experimental groups were considered significant when the P value was $< 0.05$. All statistical analyses were performed using Prism 6 statistical software (GraphPad Software, Inc. San Diego, CA, USA)

## Results

### Effects of STZ treatment in female mice

STZ treatment decreased the body weight of both WT and PAI-1 KO mice from 1 week after the final administration of STZ (Fig. 1a). Four days after STZ treatment, blood glucose levels were remarkably elevated in both WT and PAI-1 KO mice (Fig. 1b), indicating that STZ successfully induced diabetes in these mice, which was consistent with our previous study [8, 9].

Effects of diabetic state on the accumulation of neutrophils and macrophages at the damaged site after bone injury

Since neutrophils and macrophages are crucial for the inflammatory stage of bone repair, we examined the effects of diabetic state on the accumulation of these cells at the damaged site of the femur on days 2 and 4 after bone injury using immunohistochemical analysis in female WT mice. The numbers of neutrophils and macrophages were counted as the number of Gr-1- and F4/80-positive cells, respectively. No significant differences were observed between control and STZ-treated WT mice in terms of the numbers of neutrophils at the damaged site on day 2 after bone injury (Fig. 1e, f). However, the number of macrophages at the damaged site was significantly decreased on day 2, but not on day 4, after bone injury in STZ-treated mice compared with control mice, suggesting that diabetic state reduces the accumulation of macrophages at the damaged site in the inflammatory phase of bone repair in mice (Fig. 1c, d).

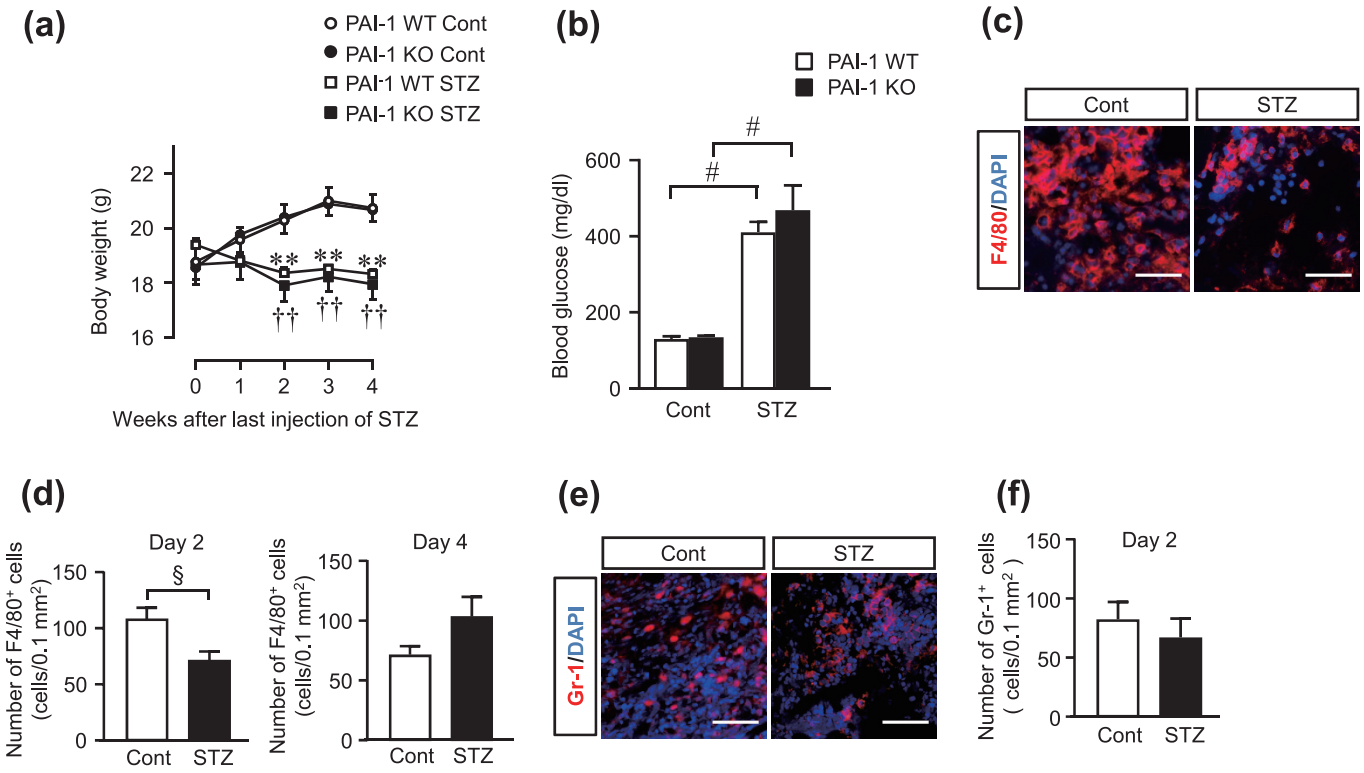


Fig. 1. Effects of streptozotocin (STZ) treatment on body weight, blood glucose levels, and numbers of macrophages and neutrophils at the damaged site after femoral bone injury in mice. (a) Growth curve during experiments in control (Cont) and STZ-treated female wild type (WT) and PAI-1-deficient (KO) mice. n=5 mice in each group. **P < 0.01 vs. WT control group, ††P <0.01 vs. PAI-1 KO control group (Tukey–Kramer test). (b) Blood glucose levels in control and STZ-treated female WT and PAI-1 KO mice. n=5 mice in each group. #P < 0.05 (Dunn test). (c, e) Photographs of F4/80-positive and Gr-1-positive cells at the damaged site on day 2 after femoral bone injury in WT mice. Scale bars indicate 50 μm. (d) Quantification of F4/80-positive cells at the damaged site on day 2 and day 4 after femoral bone injury in WT mice. n=5 WT mice in each group. §P < 0.05 (Mann-Whitney *U* test). (f) Quantification of Gr-1-positive cells at the damaged site on day 2 after femoral bone injury in WT mice. n=5 WT mice in each group. The data are expressed as means ± SEM (a, b, d, f).

Effects of diabetic state on the inflammation-related gene expression at the damaged site after bone injury

Macrophages secrete various cytokines and regulators in response to the microenvironment. Tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1$\beta$, inducible nitric oxide synthase (iNOS) and IL-6 represent M1 macrophage-synthesized factors, and IL-10, CD206, arginase 1 and transforming growth factor (TGF)-$\beta$ represent M2 macrophage-synthesized factors [12]. We examined the mRNA levels of inflammatory- and macrophage-related genes in bone tissues at the damaged site on days 2, 4 and 7 after femoral bone injury in female WT mice. STZ treatment significantly decreased the mRNA levels of macrophage colony-stimulating factor (MCSF) at the damaged site on day 2 after bone injury, although it did not affect the mRNA levels of monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1$\alpha$ or IL-4 (Fig. 2a–d). As for the markers related to the phenotypes of M1 and M2 macrophages, STZ treatment significantly decreased the mRNA levels of iNOS, IL-6 and CD206 at the damaged site on day 2 after bone injury, although the effects of STZ on the mRNA levels of TNF-$\alpha$, IL-1$\beta$, IL-10 and arginase 1 were not significant (Fig. 3a, b). On the other hand, STZ slightly enhanced TNF-$\alpha$ mRNA levels at the damaged site on day 4 after bone injury (Fig. 3a).

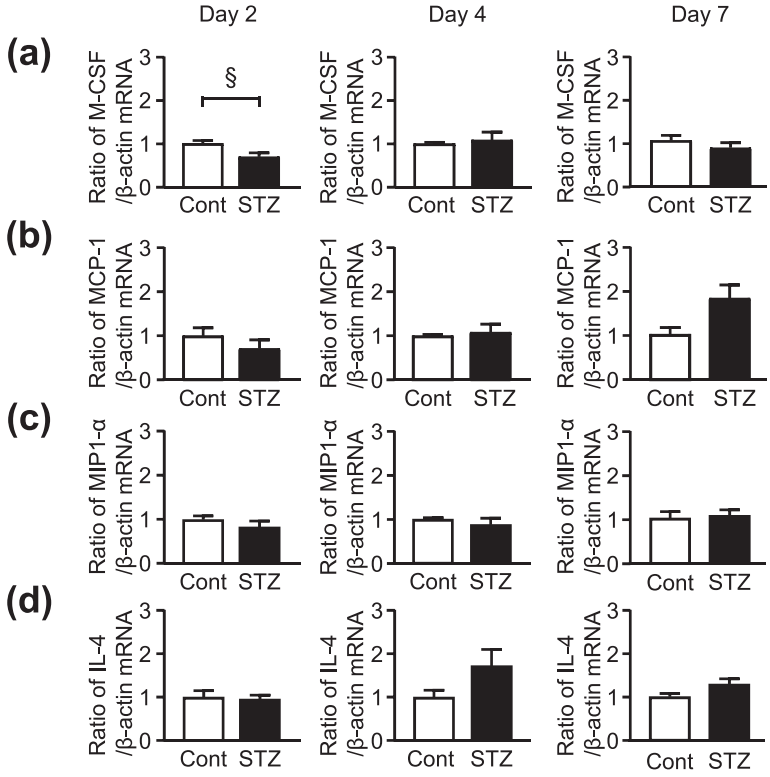


Fig. 2. Effects of diabetic state on the mRNA levels of macrophage-related factors at the damaged site on days 2, 4 and 7 after femoral bone injury in WT mice. Real time-PCR analysis of macrophage colony stimulation factor (MCSF) (a), monocyte chemoattractant protein (MCP)-1 (b), macrophage inflammatory protein (MIP)-1$\alpha$ (c), interleukin (IL)-4 (d) and $\beta$-actin mRNA at the damaged site on days 2, 4 and 7 after femoral bone injury in WT mice. The data are expressed relative to $\beta$-actin mRNA values. The data represent the mean ± SEM. n=6, (day 2, Cont) and 6 (day 2, STZ), n=7, (day 4, Cont) and 9 (day 4, STZ), n=6, (day 7, Cont) and 7 (day 7, STZ), WT mice. § $P < 0.05$ (Mann-Whitney $U$ test).

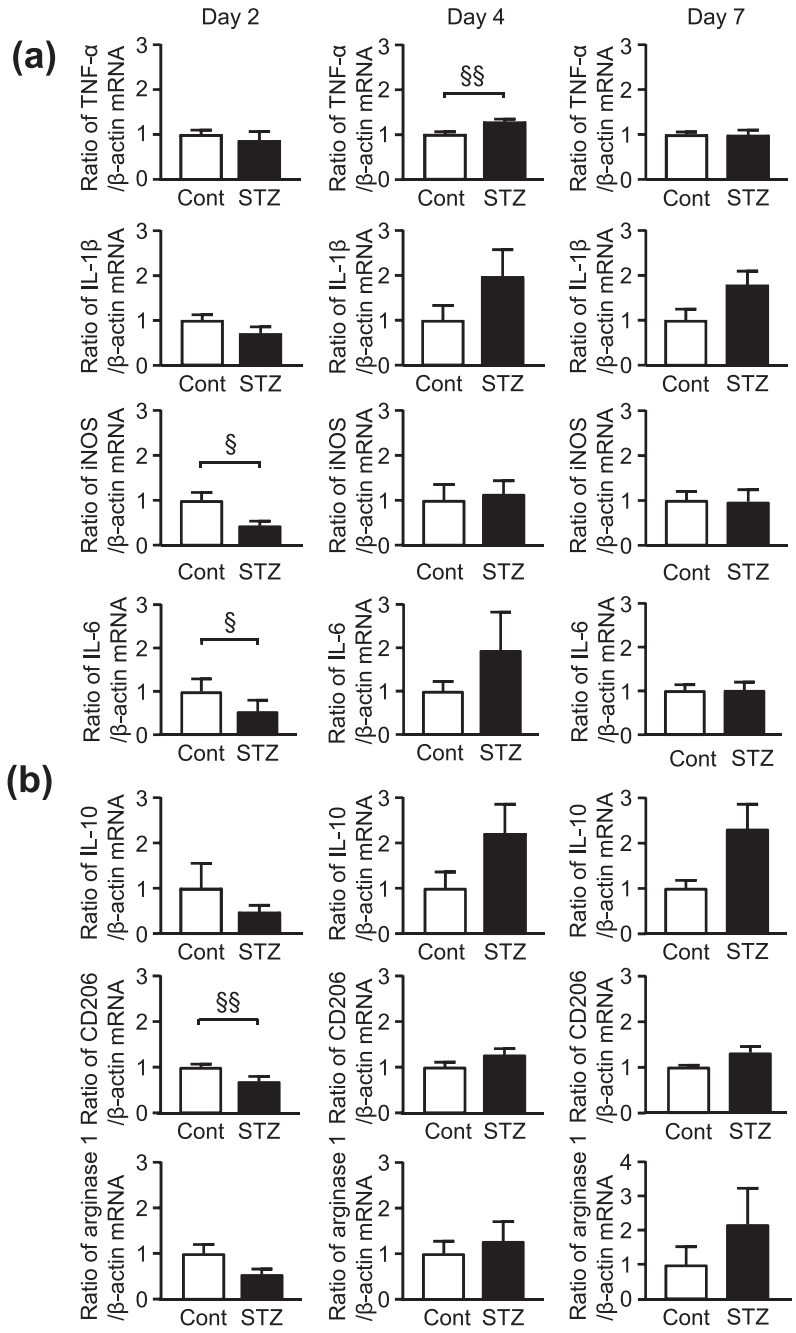


Fig. 3. Effects of diabetic state on the mRNA levels of macrophage-producing factors at the damaged site on days 2, 4 and 7 after femoral bone injury in WT mice. (a) Real time-PCR analysis of the mRNA levels of M1 macrophage-synthesized factors, including tumor necrosis factor (TNF)-$\alpha$, IL-1$\beta$, inducible nitric oxide synthase (iNOS) and IL-6, at the damaged site on days 2, 4 and 7 after femoral bone injury in WT mice. (b) Real time-PCR analysis of the mRNA levels of M2 macrophage-synthesized factors, including IL-10, CD206, arginase 1 and transforming growth factor (TGF)-$\beta$, at the damaged site on days 2, 4 and 7 after femoral bone injury in WT mice. The data are expressed relative to $\beta$-actin mRNA values. The data represent the mean ± SEM. n=6, (day 2, Cont) and 6 (day 2, STZ), n=7, (day 4, Cont) and 9 (day 4, STZ), n=6, (day 7, Cont) and 7 (day 7, STZ), WT mice. § § $P < 0.01$ and § $P < 0.05$ (Mann-Whitney $U$ test).

Effects of diabetic state on number of HSCs in bone marrow after femoral bone injury

The prevalence of HSCs in the bone marrow from damaged and contralateral intact femurs after femoral bone injury in female WT mice was evaluated by flow cytometric analysis. HSCs were defined as cells that were $CD34^-$, c-$Kit^+$, Sca-$1^+$ and $Lin^-$ ($CD34^-KSL$). As shown in Fig. 4, the number of HSCs harvested from the bone marrow of damaged femurs was significantly lower than that of HSCs from contralateral intact femurs on day 2 after femoral bone injury in mice. STZ treatment attenuated a decrease in number of HSCs induced by bone injury, although it significantly reduced the number of HSCs from intact femurs compared with the control (Fig. 4). STZ treatment significantly attenuated the reduced ratio of HSC number from the bone marrow of damaged femurs on day 2 after femoral bone injury (Ratio of HSC number in injured/intact femurs: Control, 0.81 ± 0.02; STZ, 1.03 ± 0.03; P = 0.002).

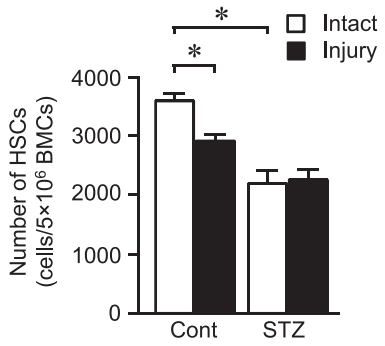


Fig. 4. Hematopoietic stem cells (HSCs) in the bone marrow from damaged femurs on day 2 after femoral bone injury in WT mice. Numbers of $CD34^-$, c-Kit+, Sca-1+, and $Lin^-$ cells ($CD34^-KSL$ cells; HSCs) harvested from the bone marrow of the contralateral intact (intact) and damaged (injury) femurs on day 2 after femoral bone injury in WT mice, as determined by flow cytometry. Data represent means ± SEM of 6 experiments (12 WT mice) in each group. *P < 0.05 (Tukey–Kramer test).

Effects of diabetic state and PAI-1 deficiency on macrophage accumulation after femoral bone injury

Since our previous study showed that PAI-1 deficiency attenuated diabetic state-induced delayed bone repair in mice [8], we examined the effects of PAI-1 deficiency on macrophage accumulation after femoral bone injury in female mice. As shown in Fig. 5, PAI-1 deficiency significantly attenuated the decrease in the number of $F4/80^+$ cells induced by STZ treatment at the damaged site on day 2 after femoral bone injury in mice, although PAI-1 deficiency and STZ treatment did not affect number of $F4/80^+$ cells on day 4 after bone injury (Fig. 5a, b).

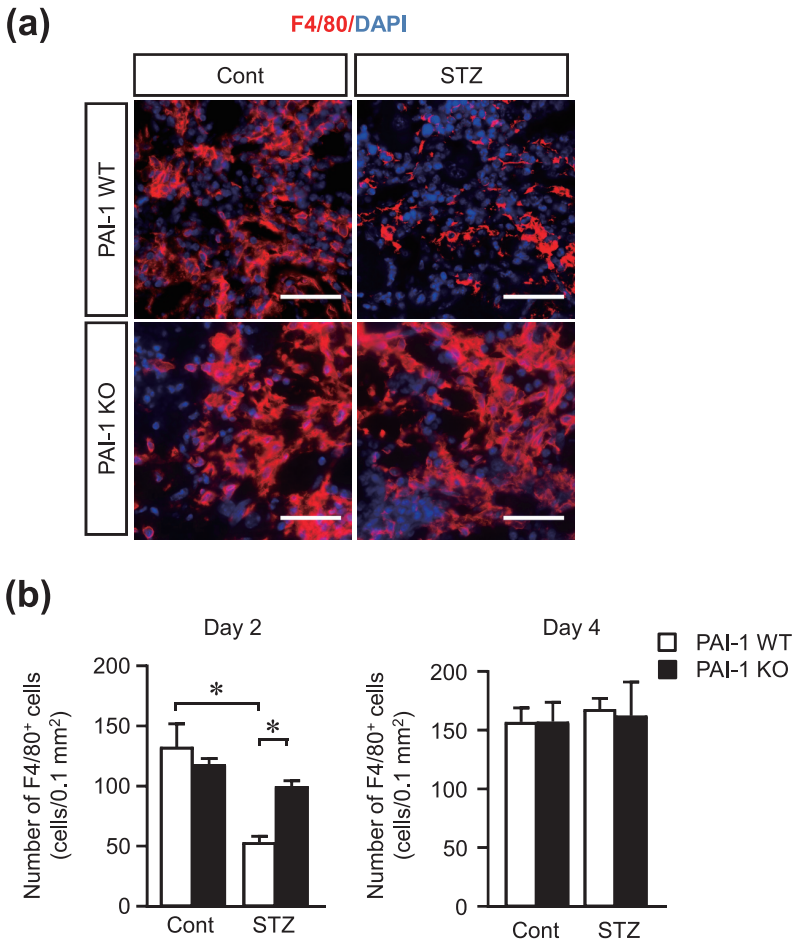


Fig. 5. Effects of PAI-1 deficiency on macrophage accumulation suppressed by diabetic state after femoral bone injury. (a) Microphotographs of F4/80 - positive cells at the damaged site on day 2 after a femoral bone injury in control and STZ-treated WT and PAI-1 KO mice. (b) Number of F4/80 - positive cells at the damaged site on days 2 and 4 after femoral bone injury in control and STZ-treated WT and PAI-1 KO mice. Scale bars indicate 50 $\mu$m. The data represent the mean ± SEM. n=5 (control WT), 6 (control PAI-1 KO), 6 (STZ-treated WT), and 5 (STZ-treated PAI-1 KO) mice (day 2). n=6 mice in each group (day 4). *P < 0.05 (Tukey–Kramer test).

Effects of diabetic state and PAI-1 deficiency on macrophage function derived from the bone marrow of the damaged femur

In order to examine the effects of diabetic state and PAI-1 deficiency on macrophage function, we evaluated the expression of macrophage-related factors in F4/80- and CD11b-double positive cells (putative macrophages) derived from bone marrow of the damaged femur in female mice on day 2 after bone injury using FACS. Although STZ treatment significantly decreased the mRNA levels of iNOS and IL-6 in F4/80- and CD11b-double positive cells from bone marrow of the damaged femur in WT mice, PAI-1 deficiency did not affect the changes in these genes induced by diabetic state (Fig. 6). STZ treatment did not affect the mRNA levels of TNF-$\alpha$, IL-1$\beta$, CD206, arginase 1 or TGF-$\beta$, in F4/80- and CD11b-double positive cells from the bone marrow of the damaged femur with or without PAI-1 deficiency, although it significantly reduced IL-10

mRNA levels in PAI-1 KO mice (Fig. 6).

Next, we examined the phagocytosis of macrophages at the damaged site on day 2 after femoral bone injury using transmission electron microscopy in WT and PAI-1 KO female mice. As shown in Fig. 7a, recruited macrophages with well-extended pseudopodia were observed at the damaged site in WT and PAI-1 KO mice. These macrophages engulfed erythrocytes or cellular debris at the damaged site in WT and PAI-1 KO mice. Although STZ treatment significantly reduced the ratio of macrophage phagocytosis at the damaged site in WT mice, PAI-1 deficiency attenuated the decrease in the ratio of macrophage phagocytosis induced by STZ treatment in mice (Fig. 7b).

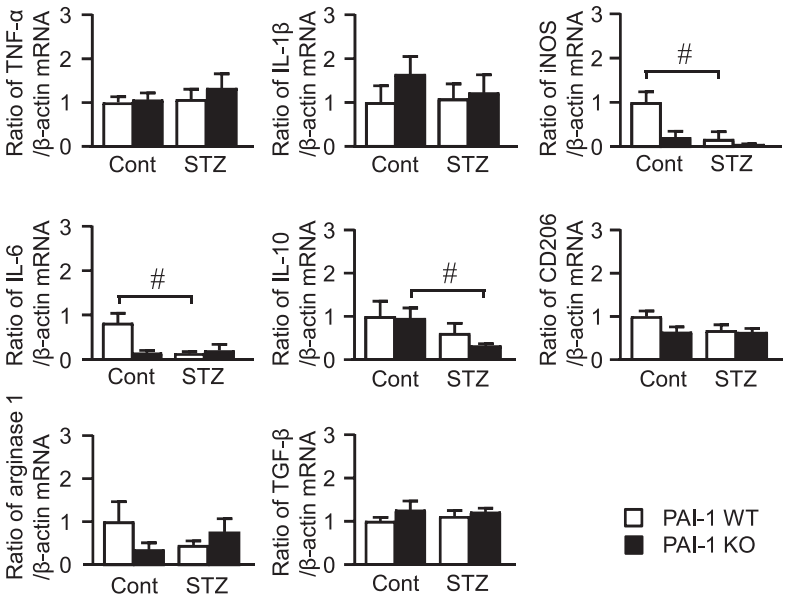


Fig. 6. Effects of PAI-1 deficiency on expression of macrophage-related factors in macrophages derived from the bone marrow of damaged femurs of control and STZ-treated WT and PAI-1 KO mice. Real time-PCR analysis of TNF-$\alpha$, IL-1$\beta$, iNOS, IL-6, IL-10, CD206, arginase 1, TGF-$\beta$ and $\beta$-actin mRNA in the femurs on day 2 after a femoral bone injury. The data are expressed relative to $\beta$-actin mRNA values. The data represent the mean ± SEM of 7 mice in each group. #$P < 0.05$ (Dunn test).

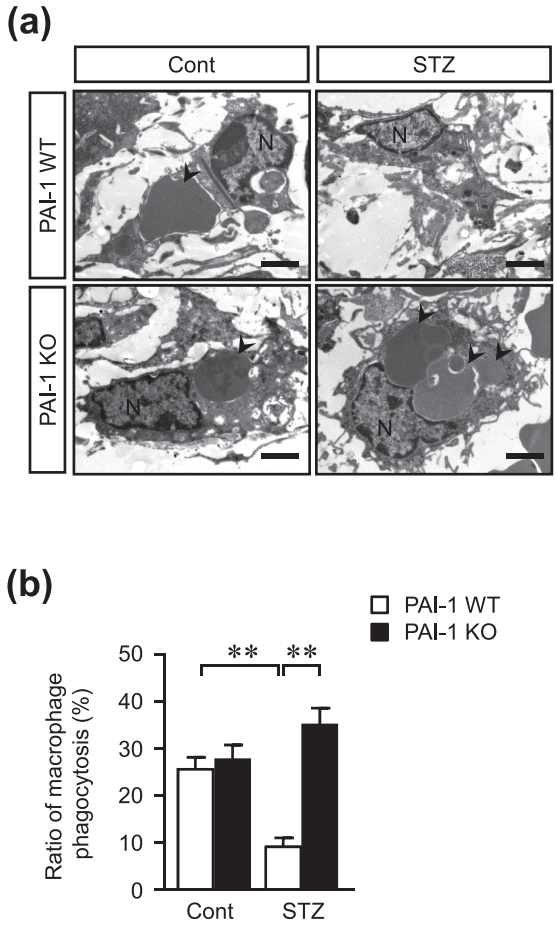


Fig. 7. Effects of diabetic state and PAI-1 deficiency on macrophage phagocytosis after femoral bone injury. (a) Transmission electron microscopic photographs of macrophages in WT and PAI-1 KO mice with or without STZ treatment at the damaged site on day 2 after femoral bone injury. The results represent experiments performed on 4 (control WT), 4 (control PAI-1 KO), 5 (STZ-treated WT) and 5 (STZ-treated PAI-1 KO) mice. Arrowheads indicate erythrocytes in macrophages. Scale bars indicate 2 $\mu$m. N: nucleus. (b) Activity of macrophage phagocytosis was evaluated by number of phagocytosing macrophages to the subject macrophages (ratio of macrophage phagocytosis) at the damaged site on day 2 after femoral bone injury assessed by transmission electron microscopy. The data represent the mean ± SEM. n=4 (control WT), 4 (control PAI-1 KO), 5 (STZ-treated WT) and 5 (STZ-treated PAI-1 KO) mice. **$P < 0.01$ (Tukey–Kramer test).

Effects of diabetic state and PAI-1 deficiency on the expression of SDF-1 on the endosteum of the damaged site after bone injury

Our previous study indicated that the induction of SDF-1 expression at the damaged site mediates the changes in the bone marrow populations of HSCs induced by bone injury in mice [27]. We therefore examined the effects of diabetic state and PAI-1 deficiency on the expression of SDF-1 in osteoblastic cells on the endosteum of the damaged site on day 2 after bone injury in female mice. We observed numerous SDF-1- and Osterix-double positive cells on the endosteum of the damaged site on day 2 after bone injury (Fig. 8a). Although STZ treatment seemed to suppress the number of SDF-1- and Osterix-double positive cells in WT

mice, PAI-1 deficiency did not alter the effects of diabetic state on SDF-1 expression (Fig. 8a, b). Moreover, PAI-1 deficiency did not affect the levels of SDF-1 mRNA in bone marrow stromal cells with or without osteoblastic differentiation with BMP-2 treatment for 24 h as well as primary mouse osteoblasts (Fig. 8c, d), suggesting that PAI-1 deficiency does not affect SDF-1 expression in mouse preosteoblasts and osteoblasts.

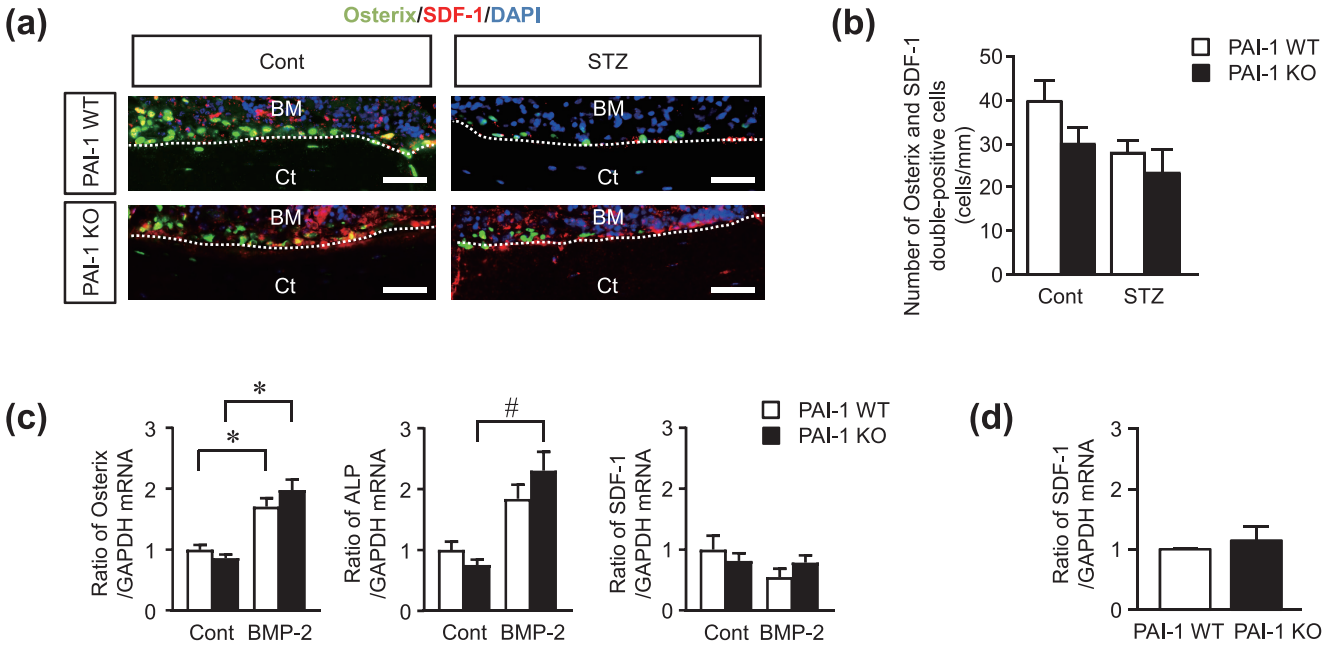


Fig. 8. Effects of PAI-1 deficiency and diabetic state on the expression of SDF-1. (a) Microphotographs of SDF-1- and Osterix-double positive cells on the endosteum of damaged site on day 2 after femoral bone injury in control or STZ-treated WT and PAI-1 KO mice. The results represent experiments performed on 5 (control WT), 6 (control PAI-1 KO), 6 (STZ-treated WT) and 5 (STZ-treated PAI-1 KO) mice. Scale bars indicate 50 $\mu$m. The dotted line indicates the border of the endosteum of cortical bone and the bone marrow space. BM: Bone marrow space. Ct: Cortical bone. (b) Numbers of SDF-1- and Osterix-double positive cells on the endosteum at the damaged site on day 2 after femoral bone injury in control or STZ-treated WT and PAI-1 KO mice. The data represent the mean ± SEM. n=5 (control WT), 6 (control PAI-1 KO), 6 (STZ-treated WT) and 5 (STZ-treated PAI-1 KO) mice. (c) Mouse bone marrow stromal cells were obtained from 8-week-old female WT and PAI-1 KO mice. Total RNA was extracted from the bone marrow stromal cells with or without osteoblastic differentiation with 100 ng/ml BMP-2 treatment for 24 h. The mRNA levels of SDF-1, Osterix, alkaline phosphatase (ALP) and GAPDH were assessed by real time-PCR. The data are mRNA levels expressed relative to GAPDH. The data represent the mean ± SEM (n=5–6). *P < 0.05 (Tukey–Kramer test). #P < 0.05 (Dunn test). (d) Total RNA was extracted from confluent primary osteoblasts. The mRNA levels of SDF-1 and GAPDH were assessed by real time-PCR. The data are mRNA levels expressed relative to GAPDH. The data represent the mean ± SEM (n=6).

Discussion

In the present study, we demonstrated that type 1 diabetic state suppressed the accumulation of macrophages as well as the expression of MCSF, IL-6, iNOS and CD206 at the damaged site after bone injury in the inflammatory phase of bone repair in female mice. PAI-1 deficiency attenuated the accumulation and phagocytosis of macrophages suppressed by diabetic state in these mice.

Neutrophils and macrophages are important cells that participate in the bone repair process, especially in the early inflammatory phase [11]. Previous studies suggest that these cells are related to delayed cutaneous wound healing induced by diabetic state [13–17, 24–26]. We first showed that type 1 diabetic state significantly decreased the number of F4/80-positive cells at the bone injury site in the early phase of bone repair in mice, although it did not affect number of Gr-1-positive cells, indicating that diabetic state suppresses the accumulation of macrophages, but not neutrophils, at the damaged site during the bone repair process in mice. Our previous study revealed that the number of HSCs decreased in the bone marrow during the femoral bone repair process in mice [27], which was consistent with the present data. This finding suggest that bone injury decreases number of HSCs in the bone marrow through the mobilization and recruitment of HSCs to the bone injury site to accelerate the bone repair process. Moreover, we showed that diabetic state attenuated the change in the HSC population induced by bone injury in mice. Several studies have suggested that diabetic state induces the mobilopathy of bone marrow stem cells that might be involved in tissue repair, vascularization and inflammation [28–31]. Since our previous study suggested that transplanted bone marrow HSCs can differentiate into macrophages and osteoclasts during the bone repair process after femoral bone injury in mice [27], diabetic state might suppress the mobilization and recruitment of HSCs in bone marrow, resulting in a decrease in macrophage accumulation at the damaged site during the early phase of the bone repair process in mice. In the present study, the number of macrophages at the damaged site on day 4 after bone injury seemed to be higher in STZ-treated mice, compared with that in control mice, although the difference was not statistically significant. We therefore cannot rule out the possibility that diabetic state might induce a delayed recruitment of macrophages at the damaged site after bone injury.

PAI-1, known as adipose tissue derived factor, is generally known as a serine protease inhibitor that suppresses plasminogen activators and fibrinolysis [32]. Plasma PAI-1 levels are elevated in diabetic animals and patients, and it exerts various cellular effects such as the regulation of cell migration, apoptosis and matrix degradation [32]. We showed previously that PAI-1 is involved in delayed bone repair and osteoporosis induced by type 1 diabetes as well as glucocorticoid-induced diabetes, osteoporosis and muscle wasting in mice [8, 9, 33, 34]. Gupta et al. reported that PAI-1 activates macrophage through Toll-like receptor-4 [35]. Moreover, a recent study suggested that monocyte adhesion to aortae is reduced in diabetic PAI-1-deficient mice [36]. On the other hand, the present study revealed that PAI-1 deficiency significantly attenuated the accumulation of macrophages decreased by diabetic state at the damaged site of femur in the early phase of bone repair process in mice. These findings suggested that PAI-1 is involved in decreased macrophage accumulation at

the injury site induced by diabetic state during the femoral bone repair process in female mice. Our previous study suggested that PAI-1 was involved in a decrease in osteoblast number and differentiation as well as delayed bone repair induced by diabetes in mice [8, 9]. Moreover, several studies indicated that macrophages are involved in the bone formation process in mice [18–21]. Therefore, the accumulation of macrophages at the damaged site after bone injury suppressed by PAI-1 might lead to decreased bone formation and subsequent delayed bone repair in diabetic state in mice.

MCSF and IL-4 are important for macrophage differentiation and proliferation, respectively [18, 37]. MCP-1 and MIP-1α are crucial chemokines for the recruitment of macrophages [37]. Macrophages can be roughly divided into two subtypes, M1 and M2. The former classically induces inflammation, and the latter is alternatively activated and associated with tissue regeneration [12, 37]. In the present study, STZ treatment significantly decreased the expression of MCSF in bone tissues at the damaged site on day 2 after bone injury in mice. This finding suggests that diabetic state decreased MCSF expression at sites of bone injury in the inflammatory stage of the bone repair process, which might lead to a decrease in macrophage accumulation into the damaged site after femoral bone injury. On the other hand, STZ treatment significantly suppressed the expression of IL-6, iNOS and CD206 in the bone tissues at the damaged site on day 2 after bone injury in mice in our data. Since the bone tissues include various cell types other than macrophages and the expression of the cytokines might not be due to the macrophage-derived one, we evaluated the expression of M1 and M2 macrophage-related factors in F4/80- and CD11b-double positive cells (putative macrophages) in the bone marrow at the damaged bone on day 2 after bone injury in mice. We revealed that STZ treatment significantly decreased the expression of iNOS and IL-6 in macrophages at the damaged site after bone injury in mice. These findings suggest that diabetic state might negatively influence the production of factors from macrophages at the damaged site in the inflammation phase during the bone repair process after bone injury in mice. However, PAI-1 deficiency did not affect the expression of those factors by macrophages at the damaged site in diabetic state in mice, suggesting that PAI-1 is not involved in the effects of diabetic state on macrophage-related factor production in macrophages at the damaged site after bone injury in mice.

SDF-1 is crucial for the regulation of the migration, maintenance and differentiation of various bone marrow cells [37]. We reported previously that SDF-1 is involved in the decrease in HSC populations in the bone marrow at the damaged site after femoral bone injury in mice [27], suggesting that bone damage induces the migration of HSCs from the bone marrow through the induction of SDF expression at the damaged site. In the present study, PAI-1 deficiency did not affect the number of SDF-1- and Osterix-double positive preosteoblasts at the endosteum after bone injury or the expression of SDF-1 in mouse primary osteoblasts, although type 1 diabetic state seemed to decrease the number of SDF-1- and Osterix-double positive preosteoblasts at the endosteum after bone injury. These findings suggest that PAI-1 is not involved in the induction of SDF-1 expression in preosteoblasts and osteoblasts at the endosteum at the damaged site after bone injury in mice. Taken together, PAI-1 might not related to changes in the number of HSCs in the bone marrow at the dam-

aged site induced by diabetic state in mice. In the present study, diabetic state decreased the expression of MCSF in the bone tissues at the damaged site on day 2 after bone injury in mice, suggesting the possibility that MCSF might be related to the effects of PAI-1 on macrophages during bone repair. The detailed mechanisms by which PAI-1 is involved in the accumulation of macrophages during the bone repair process will be explored in future studies.

Macrophages play crucial roles in the clearance of cellular debris and apoptotic and necrotic cells as well as hematoma during tissue repair. Previous studies showed that diabetic state impairs the clearance of apoptotic cells and bacteria by macrophage phagocytosis [38－40]. In the present study, PAI-1 deficiency attenuated the decrease in the ratio of macrophage phagocytosis induced by diabetic state at the damaged site after femoral bone injury in female mice. Park et al. revealed that an anti-PAI-1 antibody and PAI-1 deficiency enhanced the phagocytosis of necrotic neutrophils by macrophages, indicating that PAI-1 inhibits neutrophil efferocytosis [41]. These findings suggest that PAI-1 contributes to impaired macrophage phagocytosis induced by diabetic state at the damaged bone site. Khanna et al. revealed that impaired macrophage phagocytosis is causally related to delayed wound healing in mice [13]. Moreover, we showed previously that PAI-1 deficiency attenuated the delayed bone repair in diabetic state in mice [8]. Taken together, the suppression of macrophage phagocytosis by PAI-1 might be involved in delayed bone repair induced by diabetic state in female mice.

In conclusion, we first showed that diabetic state decreases the accumulation and phagocytosis of macrophages at a site of bone injury during the early bone repair process through PAI-1 in female mice. Macrophages and PAI-1 might be targets for the treatment of delayed bone repair in diabetic patients.

Abbreviations

AGEs, advanced glycation end products

ALP, Alkaline phosphatase

α-MEM, Minimum essential medium alpha modification

BMP, Bone morphogenetic protein

DMEM, Dulbecco's modified Eagle's medium

FBS, Fetal bovine serum

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

HSCs, Hematopoietic stem cells

IL, Interleukin

iNOS, inducible nitric oxide synthase

MCP, monocyte chemoattractant protein

MCSF, macrophage colony-stimulating factor

MIP, macrophage inflammatory protein

PAI-1, plasminogen activator inhibitor-1

PAI-1 KO, PAI-1 gene deficiency

SDF, stromal cell-derived factor

STZ, streptozotocin

TGF, transforming growth factor

TNF, tumor necrosis factor

WT, wild-type

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