Role of Macrophages and Plasminogen Activator Inhibitor-1 in Delayed Bone Repair in Diabetic Female Mice

Takeshi Shimoide,1 Naoyuki Kawao,1 Yukinori Tamura,1 Kiyotaka Okada,1 Yoshitaka Horiuchi,2 Katsumi Okumoto,2 Shinji Kurashimo,2 Masayoshi Ishida,1 Kohei Tatsumi,1 Osamu Matsuo,1 and Hiroshi Kaji1

1Department of Physiology and Regenerative Medicine, Kindai University Faculty of Medicine, Osaka-sayama 589-8511, Japan; and 2Life Science Research Institute, Kindai University, Osaka-sayama 589-8511, Japan

Delayed fracture healing is a clinical problem in diabetic patients. However, the mechanisms of diabetic delayed bone repair remain unknown. Here, we investigate the role of macrophages in diabetic delayed bone repair after femoral bone injury in streptozotocin (STZ)-treated and plasminogen activator inhibitor-1 (PAI-1)–deficient female mice. STZ treatment significantly decreased the numbers of F4/80-positive cells (macrophages) but not granulocyte-differentiation antigen-1–positive cells (neutrophils) at the damaged site on day 2 after femoral bone injury in mice. It significantly decreased the messenger RNA (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 induced by bone injury. On the other hand, PAI-1 deficiency significantly attenuated a decrease in the number of F4/80-positive 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 diabetes in mice. PAI-1 deficiency significantly attenuated the phagocytosis of macrophages at the damaged site suppressed by diabetes. In conclusion, we demonstrated that type 1 diabetes decreases accumulation and phagocytosis of macrophages at the damaged site during early bone repair after femoral bone injury through PAI-1 in female mice. (Endocrinology 159: 1875–1885, 2018)

Diabetes is characterized by chronic hyperglycemia based on impaired insulin action and secretion. Diabetic patients suffer from various complications caused by microvascular and macrovascular disease, and it is well known that cutaneous wound injury repair is delayed in diabetic patients. Recent findings suggest that fracture risk is increased mainly by 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, the mechanisms of diabetic osteoporosis and delayed bone repair remain unclear.

Several studies have 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 and osteopenia induced by streptozotocin (STZ) in female mice, although vitamin D deficiency did not affect

Abbreviations: BMP, bone morphogenetic protein; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; Gr-1, granulocyte-differentiation antigen-1; HSC, hematopoietic stem cell; iNOS, inducible nitric oxide synthase; MCP, monocyte chemoattractant protein; MCSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; mRNA, messenger RNA; PAI-1, plasminogen activator inhibitor-1; PAI-1 KO, plasminogen activator inhibitor-1 gene deficiency; PCR, polymerase chain reaction; SDF, stromal cell–derived factor; STZ, streptozotocin; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor α; WT, wild-type.
delayed bone repair induced by type 1 diabetes in mice (8–10).

It is well known that bone repair occurs in three phases: inflammation, restoration, and remodeling (11). In the inflammatory phase, immune cells, such as neutrophils and macrophages, migrate to 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 γ is involved in impaired cutaneous wound healing in type 2 diabetic mice (11). Moreover, advanced glycation end products 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 tissue repair.

As for the relationships between macrophages and bone, recent studies have indicated that a specialized resident bone macrophage population, osteomacs, plays some role in bone formation in ossification and mineralization in mice (18–21). We reported that macrophages that migrate to the site of injured bones after femoral bone injury might participate in tissue fibrinolysis–related bone repair processes in mice (22, 23). These findings suggest that macrophages migrating to bone injury sites are important for bone repair after bone injury or fracture. On the other hand, cutaneous wound healing is disrupted by an increase in neutrophil extracellular trap–driven chronic inflammation in diabetes (24, 25). Collison et al. (26) reported that the number of neutrophils extruding from capillaries is decreased by an increase in advanced glycation end products. 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 remain unknown.

In the current study, we investigated the role of macrophages and neutrophils in delayed bone repair after bone injury by 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 diabetes in female mice (8). We therefore used female mice for the current study.

### Materials and Methods

#### Materials

Anti–granulocyte-differentiation antigen-1 (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) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Bone morphogenetic protein (BMP)–2 was provided by Pfizer Inc. (Groton, CT).

#### 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, intraperitoneal, in saline) (Sigma, St. Louis, MO), a cytotoxin of pancreatic β-cells, for 4 days (9). Control mice were injected with saline alone. Four days after the last injection, nonfasting blood glucose levels were measured with a blood glucose meter (Glutest Ace; Sanwa Kagaku Kenkysyo, Nagoya, Japan) and blood from the tail vein. Mice with blood glucose levels >300 mg/dL were considered 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

### Table 1. Antibodies Used

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Antibody</th>
<th>Manufacturer, Catalog No.</th>
<th>RRID</th>
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<td>Ab_2088173</td>
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Abbreviations: ChIP, chromatin immunoprecipitation; RRID, Research Resource Identifier.
12-hour light/12-hour 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. All animal experiments were approved by the Experimental Animal Welfare Committee of 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 midfemur 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 with a drill 0.9 mm in diameter. 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°C, 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 hours, 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 with a tyramide signal amplification system (PerkinElmer, Waltham, MA). These sections were counterstained with 4',6-diamidino-2-phenylindole and photographed with the use of a fluorescence microscope (BZ-700; Keyence, Osaka, Japan).

**Real-time polymerase chain reaction**

Total RNA was isolated from a 5-mm piece of femur containing the damaged site or cells with an RNeasy Mini Kit (Qiagen, Hilden, Germany). The incorporation of SYBR Green (Applied Biosystems, Carlsbad, CA) into double-stranded DNA was assessed by real-time polymerase chain reaction (PCR) with an ABI StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers for real-time PCR are shown in Supplemental Table 1. The messenger RNA (mRNA) levels of the target genes were normalized with β-actin or glyceraldehyde 3-phosphate dehydrogenase 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 of two mice, Hanks balanced salt solution buffer with 2% fetal bovine serum (FBS) was used. Then, bone marrow cells were added to an equivalent volume of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) and were harvested by centrifugation for 15 minutes at 630g at 4°C. 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). HSCs were identified in bone marrow cell populations with 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 antilineage 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 medium (DMEM) with 1% penicillin streptomycin. The cells were labeled at 4°C for 30 minutes 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 with an 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 postfixed in the same fixative overnight at 4°C. After fixation in 1% buffered osmium tetroxide and prestaining 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 ultrathin sections were stained with 3% uranyl acetate for 20 minutes at room temperature. The stained sections were photographed with 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, 25 macrophages at the damaged site of femur were photographed in each mouse, and the number of phagocytosing macrophages with erythrocytes or cellular debris $2 \mu m$ in diameter was quantified in a blinded manner. Then, the ratio of phagocytosing macrophages to subject macrophages 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 with DMEM. After the cells were grown in DMEM with 10% FBS for 24 hours, the nonadherent cells were removed with 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 $a$ modification containing 1 mg/mL collage-nase and 0.25% trypsin for 20 minutes at 37°C. The cells from the second, third, and fourth digestions were collected and cultured in minimum essential medium $a$ modification with 10% FBS.

**Statistical analysis**

All data are represented as means ± standard error of the mean. Statistical significances were assessed with the Mann-Whitney U test for comparisons of two groups. Normality of distribution was tested with the Shapiro-Wilk test for multiple comparisons. A two-way analysis of variance followed by the Tukey-Kramer test was performed when data presented a normal distribution. The Dunn test was performed when data did not present a normal distribution for multiple comparisons. Differences between experimental groups were considered significant when the $P$, 0.05. All statistical analyses were performed in Prism 6 statistical software (GraphPad Software, Inc., San Diego, CA).

![Figure 2. Effects of diabetes 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 (a) MCSF, (b) MCP-1, (c) MIP-1$\alpha$, and (d) IL-4 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 ± standard error of the mean: n = 6 [day 2, control (Cont)] and n = 6 (day 2, STZ); n = 7 (day 4, Cont) and n = 9 (day 4, STZ); and n = 6 (day 7, Cont) and n = 7 (day 7, STZ). §$P < 0.05$ (Mann-Whitney U test).]
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. 1(a)]. Four days after STZ treatment, blood glucose levels were remarkably elevated in both WT and PAI-1 KO mice [Fig. 1(b)], indicating that STZ successfully induced diabetes in these mice, which was consistent with our previous study (8, 9).

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

Because neutrophils and macrophages are crucial for the inflammatory stage of bone repair, we examined the effects of diabetes on the accumulation of these cells at the damaged site of the femur on days 2 and 4 after bone injury by 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. 1(c) and 1(d)]. However, the number of macrophages at the damaged site was significantly lower on day 2, but not on day 4, after bone injury in STZ-treated mice compared with control mice, suggesting that diabetes reduces the accumulation of macrophages at the damaged site in the inflammatory phase of bone repair in mice [Fig. 1(e) and 1(f)].

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

Macrophages secrete various cytokines and regulators in response to

![Figure 3](https://academic.oup.com/endo/article-abstract/159/4/1875/4924487)

Figure 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 TNF-α, IL-1β, 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, and arginase 1, at the damaged site on days 2, 4, and 7 after femoral bone injury in WT mice. The data are expressed relative to β-actin mRNA values. The data represent the mean ± standard error of the mean: n = 6, [day 2, control (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; §§P < 0.05 (Mann-Whitney U test).
the microenvironment. Tumor necrosis factor α (TNF-α), interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), and IL-6 represent M1 macrophage-synthesized factors, and IL-10, CD206, arginase 1, and transforming growth factor β (TGF-β) 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)-1α, or IL-4 [Fig. 2(a)–2(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-α, IL-1β, IL-10, and arginase 1 were not significant [Fig. 3(a), 3(b)]. On the other hand, STZ slightly increased TNF-α mRNA levels at the damaged site on day 4 after bone injury [Fig. 3(a)].

**Effects of diabetes on the 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−. 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 the 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).

**Effects of diabetes and PAI-1 deficiency on macrophage accumulation after femoral bone injury**

Because our previous study showed that PAI-1 deficiency attenuated diabetes-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-positive 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 the number of F4/80-positive cells on day 4 after bone injury [Fig. 5(a) and 5(b)].

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

To examine the effects of diabetes 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 by using fluorescence-activated cell sorting. 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 diabetes (Fig. 6). STZ treatment did not affect the mRNA levels of TNF-α, IL-1β, CD206, arginase 1, or TGF-β 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 by using transmission electron microscopy in WT and PAI-1 KO female mice. As shown in Fig. 7(a), 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. 7(b)].

Effects of diabetes 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 changes in the bone marrow populations of HSCs induced by bone injury in mice (27). We therefore examined the effects of diabetes 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. 8(a)]. 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 diabetes on SDF-1 expression [Fig. 8(a) and 8(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 hours or of primary mouse osteoblasts [Fig. 8(c) and 8(d)], suggesting that PAI-1 deficiency does not affect SDF-1 expression in mouse preosteoblasts and osteoblasts.

Discussion

In the current study, we demonstrated that type 1 diabetes suppressed the accumulation of macrophages and 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 diabetes in these mice.

Neutrophils and macrophages are important cells that participate in bone repair, especially in the early inflammatory phase (11). Previous studies have suggested that these cells are related to delayed cutaneous wound healing induced by diabetes (13–17, 24–26). We showed that type 1 diabetes 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 the number of Gr-1–positive cells, indicating that diabetes suppresses the accumulation of macrophages, but not neutrophils, at the damaged site during bone repair in mice. Our previous study revealed that the number of HSCs decreased in the bone marrow during femoral bone repair in mice (27), which was consistent with the present data. This finding suggests that bone injury decreases the number of HSCs in the bone marrow by mobilizing and recruiting HSCs to the bone injury site to accelerate bone repair. Moreover,
we showed that diabetes attenuated the change in the HSC population induced by bone injury in mice. Several studies have suggested that diabetes induces the mobilopathy of bone marrow stem cells that might be involved in tissue repair, vascularization, and inflammation (28–31). Because our previous study suggested that transplanted bone marrow HSCs can differentiate into macrophages and osteoclasts during bone repair after femoral bone injury in mice (27), diabetes 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 bone repair in mice. In the current 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 diabetes 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. (35) reported that PAI-1 activates macrophages through Toll-like receptor-4. Moreover, a recent study suggested that monocyte adhesion to aortae is reduced in diabetic PAI-1-deficient mice (36). On the other hand, the current study revealed that PAI-1 deficiency significantly attenuated the accumulation of macrophages decreased by diabetes at the damaged site of femur in the early phase of bone repair in mice. These findings suggested that PAI-1 is involved in decreased macrophage accumulation at the injury site induced by diabetes during femoral bone repair in female mice. Our previous study suggested that PAI-1 was involved in a decrease in osteoblast number and differentiation and delayed bone repair induced by diabetes in mice (8, 9). Moreover, several studies indicated that macrophages are involved in bone formation in mice (18–21). Therefore, 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 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 current 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 diabetes decreased MCSF expression at sites of bone injury in the inflammatory stage of bone repair, which might lead to a decrease in macrophage accumulation at 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. Because the bone tissues include various cell types other than macrophages, and the expression of the cytokines might not be caused by 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 found 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 diabetes might decrease the production of factors from macrophages at the damaged site in the inflammation phase during bone repair 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 mice, suggesting that PAI-1 is not involved in the effects of diabetes on macrophage-related factor production in macrophages at the damaged site after bone injury in mice.

SDF-1 is crucial in regulating 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 bone marrow at the damaged site after femoral bone injury in mice (27), suggesting that bone damage induces migration of HSCs from the bone marrow through the induction of SDF expression at the damaged site. In the current 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 diabetes 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 after bone injury in mice. Therefore, PAI-1 might not be related to changes in the number of HSCs in the bone marrow at the damaged site induced by diabetes in mice. In the current study, diabetes decreased the expression of MCSF in bone tissues at the damaged site on day 2 after bone injury in mice, suggesting 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 bone repair will be explored in future studies.

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

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

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Correspondence: Hiroshi Kaji, MD, PhD, Department of Physiology and Regenerative Medicine, Kindai University Faculty of Medicine, 377-2 Ohnohigashi, Osakasayama, Osaka 589-8511, Japan. E-mail: hkaji@med.kindai.ac.jp.

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