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Roles of plasminogen in the alterations in bone marrow hematopoietic stem cells during bone repair

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ABSTRACT

We previously revealed that stromal cell-derived factor-1 (SDF-1) is involved in the changes in the number of bone marrow stem cells during the bone repair process in mice. Moreover, we reported that plasminogen (Plg) deficiency delays bone repair and the accumulation of macrophages at the site of bone damage in mice. We investigated the roles of Plg in the changes in bone marrow stem cells during bone repair. We analyzed the numbers of hematopoietic stem cells (HSC) and mesenchymal stem cells (MSCs) within bone marrow from Plg-deficient and wild-type mice after a femoral bone injury using flow cytometric analysis. Plg deficiency significantly blunted a decrease in the number of HSCs after bone injury in mice, although it did not affect an increase in the number of MSCs. Plg deficiency significantly blunted the number of SDF-1- and Osterix- or SDF-1- and alkaline phosphatase-double-positive cells in the endosteum around the lesion as well as matrix metalloprotainase-9 (MMP-9) activity and mRNA levels of SDF-1 and transforming growth factor–β (TGF-β) elevated by bone injury. TGF-β signaling inhibition significantly blunted a decrease in the number of HSCs after bone injury. The present study showed that Plg is critical for the changes in bone marrow HSCs through MMP-9, TGF-β, and SDF-1 at the damaged site during bone repair in mice.

1. Introduction

The bone repair process is divided into three phases, including inflammation, repair, and remodeling (Claes et al., 2012). In the acute inflammation phase, leukocytes, including neutrophils and macrophages, are recruited into the damaged site and release cytokines and growth factors. In the repair phase, angiogenesis and endochondral ossification occur in the damaged bone. In the remodeling phase, osteoblasts and osteoclasts participate in bone formation and resorption, respectively. Local ischemia, recruitment of stem cells, and interaction among inflammatory cells are observed in the inflammation phase (Khosla et al., 2008). The cells participating in the bone repair process are derived from bone marrow in the area surrounding the injury site (Gerstenfeld et al., 2003; Nistala et al., 2010). However, the roles of bone marrow stem cells in the bone repair process after bone destruction or fractures have remained unclear. We previously revealed that the numbers of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) in bone marrow were decreased and increased in the damaged site 2 days after femoral bone injury in mice, respectively (Okada et al., 2016). That study indicated that stromal cell-derived factor-1 (SDF-1) is involved in the changes in the number of bone marrow stem cells during the bone repair process.

Plasminogen (Plg), a zymogen of the fibrinolytic enzyme, is responsible for fibrin thrombolysis by its active-type plasmin in the body (Collen and Lijnen, 1991). Conversion of Plg to plasmin is promoted by two-types of plasminogen activators, urokinase-type and tissue-type plasminogen activators (u-PA and t-PA). Acting in concert with other proteases, active plasmin is supposed to play a role in the degradation of the extracellular matrix in the context of physiological and pathological tissue remodeling and cell migration events, such as wound healing (Singer and Clark, 1999), angiogenesis (Eming et al., 2007), and tumor cell invasion (Bugge et al., 1996). We previously revealed that Plg deficiency delays bone repair and the accumulation of macrophages at the site of bone damage in mice (Kawao et al., 2013). Moreover, we revealed that t-PA and u-PA are related to the bone repair process in mice (Kawao et al., 2014, 2015). These findings suggest that the tissue fibrinolytic system is crucial for the bone repair process. Although our findings suggested that bone marrow HSCs and MSCs

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participate in the early phase of the bone repair process in mice (Okada et al., 2016), the relationships between the tissue fibrinolytic system and bone marrow stem cells during bone repair have remained unknown.

In the present study, we therefore investigated the roles of Plg in changes in the HSCs and MSCs populations in bone marrow during the bone repair process in mice and clarify its mechanism using Plg-deficient mice.

2. Materials and methods

2.1. Materials

Anti-Osterix and alkaline phosphatase (ALP) antibodies were obtained from Abcam (Cambridge, UK) and Abnova (Taipei, Taiwan), respectively. Violet (V)450/brilliant violet (BV)421-conjugated anti-CD29, Alexa 700-conjugated anti-CD34, allophycocyanin (APC)-Cy7-conjugated anti-CD44, V500/BV510-conjugated anti-CD45, APC-conjugated anti-CD73, phycoerythrin (PE)-conjugated anti-CD105, BV711-conjugated anti-c-Kit, PE-Cy7-conjugated anti-Sca-1 antibodies, and the peridinin chlorophyll protein complex (PerCp)-Cy5.5-conjugated anti-lineage antibodies cocktail (anti-CD3e, anti-CD11b, anti-B220/CD45R, anti-Gr1, and anti-TER-119 antibodies 1) were obtained from BD Biosciences (San Jose, CA, USA). SB431542 was obtained from Sigma-Aldrich (Tocric Cookson Ltd., Bristol, UK). Recombinant human bone morphogenetic protein (BMP)-2 was provided by Pfizer Inc. (Groton, CT, USA).

2.2. Animals

Male and female mice with plasminogen gene deficiency ($Plg^{-/-}$) and their wild-type counterparts ($Plg^{+/+}$), provided initially by Prof. D. Collen (University of Leuven, Leuven, Belgium), each weighing 18 to 25 g and 6 to 10 weeks old, with a mixed C57BL/6 J (75%) and 129/SvJ (25%) background, were used. All experiments were performed according to the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kindai University. The protocol was approved by the Experimental Animal Welfare Committee of Kindai University (permit no. KAME-27-025).

2.3. Murine bone damage model

Bone injury was induced in mice according to a method previously described (Okada et al., 2016). Briefly, under anesthesia induced by pentobarbital sodium (50 mg/kg, intraperitoneally), the anterior skin over the mid-femur of the right leg was incised longitudinally 5 mm in length. After splitting the muscle, the surface of the femoral bone was exposed. Thereafter, a hole was made using a drill with a diameter of 0.9 mm. The hole was irrigated with saline to prevent thermal necrosis of the margins. The incised skin was then sutured in a sterile manner, and anesthesia was discontinued.

2.4. Flow cytometric analysis

Bone marrow stromal cells were obtained from mice as previously described (Okada et al., 2016). HBSS buffer with 2% fetal bovine serum (FBS) was used to flush the bone marrow cells from the harvested femur. Bone marrow cells were then added in an equivalent volume to Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and were harvested by centrifugation for 15 min at 630 $\times g$ at 4 °C. Cells were re-suspended in PBS supplemented with 3% FBS.

Bone marrow cells were analyzed using a FACS Aria II cell sorter (BD Biosciences), as previously described (Okada et al., 2016). HSCs and MSCs were identified in bone marrow cell populations using color-conjugated antibodies specific for CD34, c-Kit, Sca-1, and CD29, CD34, CD44, CD45, CD73, CD105, Lin, respectively (Okada et al., 2016). The numbers of HSCs and MSCs harvested from the bone marrow of the contralateral intact and damaged femurs 2 days after femoral bone injury, as enumerated by flow cytometry, were measured. The results represent experiments performed on 5 mice in each group.

2.5. Histological analysis

The mice were anesthetized using pentobarbital sodium (50 mg/kg, intraperitoneally) on day 0, day 1, day 2, day 4, or day 7 after surgery. Femurs were removed, fixed in 4% paraformaldehyde, demineralized in 22.5% formic acid and 340 mM sodium citrate solution for 24 h, and embedded in paraffin. Immunostaining was performed as previously described (Okada et al., 2016). Briefly, the sections were incubated with anti-ALP antibody at a dilution of 1:100, anti-Osterix antibody at a dilution of 1:200, or anti-SDF-1 antibody at a dilution of 1:200 followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Positive signals were visualized using the tyramide signal amplification system (PerkinElmer, Waltham, MS, USA), and sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and photographed using a fluorescence microscope (E800; Canon, Tokyo, Japan) with a CCD camera or a confocal microscope (C2 Si; Nikon, Tokyo, Japan).

2.6. Quantitative real-time PCR

Total RNA was isolated from the tissues and 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) as previously described (Kawao et al., 2013). The PCR primers are listed in Table 1. The mRNA levels of the target genes were normalized with the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

2.7. Preparation of primary osteoblasts and bone marrow stromal cells

Calvarial osteoblasts were obtained from $Plg^{+/+}$ and $Plg^{-/-}$ mice according to a previously described method (Kawao et al., 2013). Briefly, calvaria was removed from 3-day-old mice, cleaned to remove soft tissue, and digested four times with 1 mg/ml collagenase and 0.25% trypsin for 20 min at 37 °C with gentle agitation. The cells from the second, third, and fourth digestions were plated and grown in Eagle's minimum essential medium (α-MEM) with 10% FBS. The cells

Table 1
Primers used for real-time PCR experiments.

Gene	Primer sequence
SDF-1	Forward 5′-CTGTGCCCTTCAGATTGTTG-3′
	Reverse 5′-TCAGCCTTCCTCGGGGGTCT-3′
TGF-β1	Forward 5′-CCTCTGTCACCTGCTCAACA-3′
	Reverse 5′-GATGAATTGGCGTGGAATCT-3′
PDGF	Forward 5′-CAGTGACCTTGGAGGACCAC-3′
	Reverse 5′-GAATGGTCACCCGAGCTTGA-3′
FGF-2	Forward 5′-CCTTGCTATGAAGGAAGATGG-3′
	Reverse 5′-TCCGTGACCGGTAAGTATTGT-3′
HGF	Forward 5′-CACCCCTTGGGAGTATTGTG-3′
	Reverse 5′-GGGACATCAGTCTCATTCACAG-3′
Osterix	Forward 5′-AGCGACCACTTGAGCAAACAT-3′
	Reverse 5′-GCGGCTGATTGGCTTCTTCT-3′
ALP	Forward 5′-ATCTTTGGTCTGGCTCCCATG-3′
	Reverse 5′-TTTCCCGTTCACCGTCCAC-3′
GAPDH	Forward 5′-AGGTCGGTGTGAACGGATTTG-3′
	Reverse 5′-GGGGTCGTTGATGGCAACA-3′

SDF-1, stromal-derived factor-1; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.