



Role of CXCR2 in the Ac-PGP-Induced Mobilization of Circulating Angiogenic Cells and its Therapeutic Implications

YANG WOO KWON,^a SEUNG JUN LEE,^b SOON CHUL HEO,^a TAE WOOK LEE,^a GYU TAE PARK,^a JUNG WON YOON,^a SEUNG-CHUL KIM,^c HO JIN SHIN,^d SANG CHUL LEE,^e JAE HO KIM^{id a,f}

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^aDepartment of Physiology, Pusan National University School of Medicine,

Yangsan, Republic of Korea; ^bDepartment of Orthopaedic Surgery, Pusan National University School of Medicine, Yangsan, Republic of Korea;

^cDepartment of Obstetrics and Gynecology, Pusan National University School of Medicine, Yangsan, Republic of Korea; ^dDivision of Hematology-Oncology,

Pusan National University School of Medicine, Yangsan, Republic of Korea; ^eFunctional Genomics Research Center, KRIBB,

Daejeon, Republic of Korea; ^fResearch Institute of Convergence Biomedical Science and Technology,

Pusan National University Yangsan Hospital, Yangsan, Republic of Korea

Correspondence: Jae Ho Kim, Ph.D., Department of Physiology, Pusan National University School of Medicine, Yangsan 50612, Gyeongsangnam-do, Republic of Korea. Telephone: 82-51-510-8073; e-mail: jhkimst@pusan.ac.kr

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ABSTRACT

Circulating angiogenic cells (CACs) have been implicated in the repair of ischemic tissues, and their mobilization from bone marrow is known to be regulated by the activations of chemokine receptors, including CXCR2 and CXCR4. This study was conducted to investigate the role of N-acetylated proline-glycine-proline (Ac-PGP; a collagen-derived chemotactic tripeptide) on CAC mobilization and its therapeutic potential for the treatment of peripheral artery diseases. Ac-PGP was administered daily to a murine hind limb ischemia model, and the effects of Ac-PGP on blood perfusion and CAC mobilization (Sca1⁺Flk1⁺ cells) into peripheral blood were assessed. Intramuscular administration of Ac-PGP significantly improved ischemic limb perfusion and increased limb salvage rate by increasing blood vessel formation, whereas Ac-PGP-induced blood perfusion and angiogenesis in ischemic limbs were not observed in CXCR2-knockout mice. In addition, Ac-PGP-induced CAC mobilization was found to occur in wild-type mice but not in CXCR2-knockout mice. Transplantation of bone marrow from green fluorescent protein (GFP) transgenic mice to wild-type mice showed bone marrow-derived cells homed to ischemic limbs after Ac-PGP administration and that GFP-positive cells contributed to the formation of ILB4-positive capillaries and α smooth muscle actin (α -SMA)-positive arteries. These results suggest CXCR2 activation in bone marrow after Ac-PGP administration improves blood perfusion and reduces tissue necrosis by inducing CAC mobilization. These findings suggest a new pharmaceutical basis for the treatment of critical limb ischemia. *STEM CELLS TRANSLATIONAL MEDICINE* 2019;8:236–246

SIGNIFICANCE STATEMENT

Critical limb ischemia is a serious form of peripheral artery disease with a 12% amputation rate within 1 year of diagnosis. Due to the angiogenesis-stimulating ability of stem cells, stem cell-based therapies are considered to offer a promising means of treating patients with ischemic disease. This study demonstrates that a collagen-derived tripeptide, N-acetylated proline-glycine-proline, stimulates blood perfusion and prevents limb amputation by promoting mobilization of circulating angiogenic cells through a CXCR2-dependent mechanism. This work suggests that Ac-PGP-CXCR2 axis offers a unique opportunity to develop a circulating angiogenic cell-mediated therapy for peripheral artery diseases.

INTRODUCTION

Peripheral arterial disease is estimated to affect 4.2%–35% of the general population in an age, gender, and smoking habit dependent manner, and critical limb ischemia, exhibiting pain at rest, accompanied by necrosis and ulceration leading to amputation has been reported to occur in up to 9.6% of those affected [1]. Peripheral arterial disease has different stages and presentations, including diabetic foot, atherosclerotic obliterans, Buerger's disease, erectile dysfunction, and

cerebral stroke [2]. Patients with diabetic peripheral arterial disease are at highest risk of progression to critical limb ischemia, and are 10 times more likely to require amputation and 20–30 times more likely to develop gangrene than the general population [3]. In affected patients, therapeutic angiogenesis is important for providing blood to ischemic tissues and for facilitating tissue regeneration after critical ischemia [4, 5].

Due to the angiogenesis-stimulating ability of stem cells, stem cell-based therapies are considered to offer a promising means of

treating patients with ischemic disease [6]. Accumulating evidence suggests circulating angiogenic cells (CACs) or endothelial progenitor cells are essentially required for vascular repair and regeneration [7–9]. CACs have been shown to express several markers, such as CD34, CD133, and Flk1 [9]. In addition, stem cell antigen 1 (Sca1) and vascular endothelial growth factor receptor type 2 (Flk1)-double positive cells have been considered as CACs [10, 11]. However, studies to purify and characterize CACs from peripheral blood (PB) have been hampered by the absence of CAC-specific markers and the heterogeneity of CACs. The CACs reportedly exhibited endothelial colony-forming activity in vitro and contributed to vasculogenesis in vivo [7, 12]. Therefore, not only characterization of surface markers but also determination of endothelial colony-forming ability is required for quantitation of CACs in peripheral blood.

CACs have been shown to integrate into blood vessels and stimulate neovascularization in ischemic limbs and hearts in animal models of hind limb ischemia and cardiac infarction [13, 14], and thus, the mobilization and recruitment of stem/progenitor cells are considered to be critical for ischemia-induced neovascularization. However, endogenous CACs of patients at highest cardiovascular risk are present in low numbers and have poor migratory and mobilization capacities, and thus, the use of autologous CACs for neovascularization was suggested to be likely to be less than effective in clinical practice [15]. For this reason, it would appear the mobilization of CACs to specific target sites might increase their therapeutic efficacies in ischemic diseases. More specifically, the mobilizations of CACs in response to various cytokines and chemokines, including granulocyte colony stimulating factor (G-CSF) [16], stromal cell-derived factor-1 α [17], and nitric oxide [18] have been shown to enhance the regeneration of ischemic tissues [19]. Of these cytokines and chemokines, G-CSF is currently regarded to induce CAC mobilization and to have therapeutic benefit in ischemic disease [20, 21]. However, in several clinical trials, G-CSF was consistently observed to increase chest pain, in-stent restenosis, and acute coronary syndrome in patients with acute myocardial infarction, probably because of its proinflammatory and procoagulant properties [22, 23]. Therefore, new drugs that stimulate the mobilization of CACs are needed for the treatment of ischemic diseases.

N-acetylated proline-glycine-proline (Ac-PGP) is a tripeptide generated from collagen by matrix metalloproteinase and prolyl endopeptidase [24], and the activation of matrix metalloproteinase and concomitant degradation of collagen lead to the increased production of Ac-PGP in brain after ischemic stroke [25]. Accumulating evidence suggests the involvement of CXCR1/2 in the cellular responses induced by Ac-PGP [26–28], which has been reported to stimulate neutrophil chemotaxis by mediating CXCR2 [26], and that Ac-PGP exhibits therapeutic effects against sepsis [27]. Ac-PGP also promotes the migration of cartilage endplate stem cells and induces their differentiation toward a proinflammatory and catabolic phenotype via CXCR1/2 [28]. We recently reported Ac-PGP accelerates neovascularization and wound repair by promoting the migration and engraftment of exogenously transplanted human cord blood-derived endothelial progenitor cells via a CXCR2-dependent mechanism [29]. However, it remains unclear whether the therapeutic effects of Ac-PGP in

peripheral artery diseases involve the mobilization of endogenous CACs from bone marrow.

To explore the possible therapeutic use of Ac-PGP for the regeneration of injured tissues, we investigated its effects on ischemic tissue repair, angiogenesis, and CAC mobilization from bone marrow in a murine ischemic hind limb model, and the role played by CXCR2 in Ac-PGP-stimulated tissue repair and angiogenesis in vivo.

MATERIALS AND METHODS

Animals

CXCR2 knockout (BALB/C-*Cxcr2*^{tm1Mwm}) mice, BALB/C wild-type mice, and C57BL/6-Tg[CAG-EGFP]10sb/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6J wild-type male mice (8- to 10-week old; 22–25 g) were purchased from Orient, Co., Ltd. (Gapyeong, Republic of Korea). All animals were housed in an air-conditioned facility (22°C–25°C; relative humidity 50%–65%) and provided a laboratory diet and water. Animal treatment and maintenance were performed in accordance with the Principles of Laboratory Animal Care, and animal experiments were performed using protocols approved by the Pusan National University Institutional Animal Use and Care Committee (PNU-2016-1381).

Murine Hind Limb Ischemia Animal Model

The hind limb ischemia mouse model was conducted by ligating and burning the artery at the hind limb as described previously [30]. All mice were anesthetized with an intraperitoneal injection of 400 mg/kg of 2,2,2-tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO) for femoral artery resection and laser Doppler perfusion imaging (LDPI). The fur was completely shaved from both hind limbs to facilitate measurements of limb perfusion. One femoral artery per animal was excised from its proximal origin as a branch of the external iliac artery to its distal bifurcation into the saphenous and popliteal arteries. Immediately after surgery, medial thighs of ischemic hind limbs were injected with Ac-PGP (1 μ M) in Hank's balanced salt solution (HBSS) buffer into four sites (20 μ l per each site) of gracilis muscle. Subsequently, Ac-PGP or HBSS buffer was injected daily into four sites of hind limb muscle for 4 weeks. Mice were carefully monitored post operation for 3 days, and Buprenex analgesic at a dose of 0.1 mg/kg body weight was injected twice a day to relieve postoperative pain.

Measurements of Blood Flow and Tissue Necrosis

Blood flows in ischemic and normal limbs were measured using a LDPI analyzer (Moor Instruments, Ltd., Devon, U.K.) on days 0, 7, 14, 21, and 28 after surgery. Contralateral hind limbs served as internal controls. Perfusions in ischemic and contralateral limbs were calculated by counting red and blue colored histogram pixels, which indicated high and low perfusion, respectively. Blood perfusions are presented as LDPI indices (defined as the ratio of ischemic limb blood flow versus nonischemic contralateral limb blood flow). Hind limb necrosis severity scores were recorded on day 28 after surgery (0 = limb salvage; 1 = toe amputation; 2 = foot amputation; and 3 = limb amputation).

Bone Marrow Transplantation

To prepare bone marrow, femurs and tibiae were collected from donor mice (6 weeks old) and bone marrow were flushed out using medium (RPMI 1640 containing 2% FBS, 10 U/ml of heparin and antibiotics). Clumps of cells were agitated and the cell suspension was then passed through a 23-gauge needle and a 40 μ m cell strainer. Cells were then centrifuged at 1,000g for 4 minutes at 4°C and resuspended in serum-free medium at a concentration of $1 \times 10^6/100 \mu$ l. For bone marrow transplantation, age-matched C57BL/6J wild-type mice were irradiated with a lethal dose of 10 Gy, and then injected intravenously with 5×10^6 donor bone marrow cells isolated from green fluorescent protein (GFP)-transgenic mice (C57BL/6-Tg[CAG-EGFP]10sb/J). Transplanted bone marrow was allowed to regenerate *in vivo* for 4 weeks before subsequent experimental procedures.

Immunofluorescence Staining

For the immunostaining study, hind limb muscles were removed, formalin-fixed, paraffin embedded, and sectioned at 5 μ m. Blood vessels were stained with rat anti-CD31 (BD), biotinylated-ILB4, rabbit anti- α -SMA or anti-CXCR2 (Abcam Plc., Cambridge, MA) antibodies, and sections were then incubated with Alexa 488 streptavidin, Alexa 488, or Alexa 568 goat anti-rat secondary antibodies (Life Technologies, Carlsbad, CA), washed, and mounted in Vectashield medium containing DAPI (a nuclear stain). Sections were then observed under a laser scanning confocal microscope (Olympus Fluoview FV1000). Bone marrow-derived cells were identified in the ischemic muscles of wild-type mice transplanted with bone marrow from GFP transgenic mice by staining with anti-GFP antibody and Alexa 488-conjugated anti-goat IgG secondary antibodies (both from Thermo Fisher Scientific, Waltham, MA). Bone marrow-derived endothelial cells were quantified by counting the number of cells positive for both GFP expression and ILB4 staining and α -SMA positive smooth muscle cells by counting the number of cells positive for GFP and α -SMA in tissue sections. Capillaries densities and numbers of arterioles/arteries were counted by identifying ILB4-, CD31- or α -SMA-positive vascular structures in three high power fields. Four randomly selected microscopic fields from three serial sections in each tissue block were examined per limb by two independent observers unaware of experimental conditions.

Isolation and Characterization of Peripheral Blood Mononuclear Cells

Ac-PGP (1 μ M) in HBSS buffer was daily administered to ischemic limbs by intramuscular injection. On day 3 after administration, peripheral blood was obtained by heart puncture and separated by Histopaque-1083 (Sigma-Aldrich Corp., St. Louis, MO, www.sigmaaldrich.com) density gradient centrifugation. To prevent clotting, the syringes, needles, and tubes used for cardiac puncture were pretreated with a solution of 2 U/ml heparin. Buffy-coat mononuclear cells (MNCs) were collected and red blood cells were lysed with RBC lysis solution (Qiagen, Valencia, CA).

Flow Cytometry Analysis of CACs

Mouse MNCs derived from peripheral blood were preincubated with Mouse BD Fc Block purified anti-mouse CD16/CD32 antibodies to block nonantigen-specific binding of immunoglobulins

to Fc receptors. The population of Sca1⁺Flk1⁺ CACs in the MNCs was determined by flow cytometry analysis as previously described [10]. PE-conjugated Flk1 or APC-conjugated Sca1 antibodies were added directly to preincubated MNCs in the presence of Mouse BD Fc Block for 30 minutes at 4°C. Isotype-matched rat IgG2a was used as a negative control. All antibodies used for flow cytometric analysis were purchased from BD Biosciences. Ten thousand events were acquired on a flow cytometry can flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using FACS Diva ver. 6.1.3 (BD Biosciences) or FlowJo ver. 10 (FlowJo, LLC, Ashland, OR). Surface molecule numbers were derived from calibration curves after subtracting the negative isotype control values.

Colony Forming Assay

Peripheral blood-derived MNCs (2×10^5 cells) were cultured for 12 days in methyl cellulose-containing medium M3236 (Stemcell Technologies, Vancouver, Canada; Center Valley, PA, www.olympusamerica.com) containing 20 ng/ml of stem cell factor, 50 ng/ml vascular endothelial growth factor, 20 ng/ml interleukin-3, 50 ng/ml basic fibroblast growth factor, 50 ng/ml epidermal growth factor receptor, and 50 ng/ml insulin-like growth factor-1. All supplementary growth factors were purchased from PeproTech (Rocky Hill, NJ, www.peprotech.com). The endothelial phenotypes of the colonies were confirmed by high uptake of acetyl LDL (DiI acetylated low-density lipoprotein [DiI-Ac-LDL], Biomedical Technologies, Stoughton, MA, www.btiinc.com) and cytochemical positivity for isolectin B4 (ILB4; Vector Laboratories, Burlingame, CA, vectorlabs.com).

Statistical Analysis

All statistical analyses were performed using SigmaPlot (version 13, Systat Software, Inc., San Jose, CA). The results of multiple observations are presented as mean \pm SD. Student's two-tailed unpaired *t* test was used to determine statistical significance of two groups. For multivariate data analysis, group differences were assessed with one-way or two-way analysis of variance, followed by Scheffé's post hoc test. Data were considered statistically significant at $p < .05$, unless indicated otherwise.

RESULTS

Ac-PGP Stimulated Angiogenesis and Alleviated Ischemic Tissue Damage in the Murine Ischemic Hind Limb Model

To determine whether Ac-PGP improves angiogenesis *in vivo*, we examined its effects on blood perfusion and tissue repair in an ischemic hind limb animal model. After removing femoral arteries, varying concentrations of Ac-PGP were administered intramuscularly into ischemic limbs. Blood flows were measured for 4 weeks using LDPI ratios as described above. Ac-PGP injections into ischemic limbs significantly increased blood perfusion, as compared with HBSS-treated controls (Fig. 1A, 1B), and the effect of Ac-PGP on blood perfusion peaked at a concentration of 1 μ M. In addition, 1 μ M Ac-PGP injections alleviated tissue necrosis and the need for amputation (as demonstrated by reduced necrosis scores at 4 weeks after the induction of ischemia) as compared with HBSS administered controls (Fig. 1C).

Angiogenesis is crucial for ischemic tissue repair and for the recovery of blood perfusion [31]. To evaluate the effect of

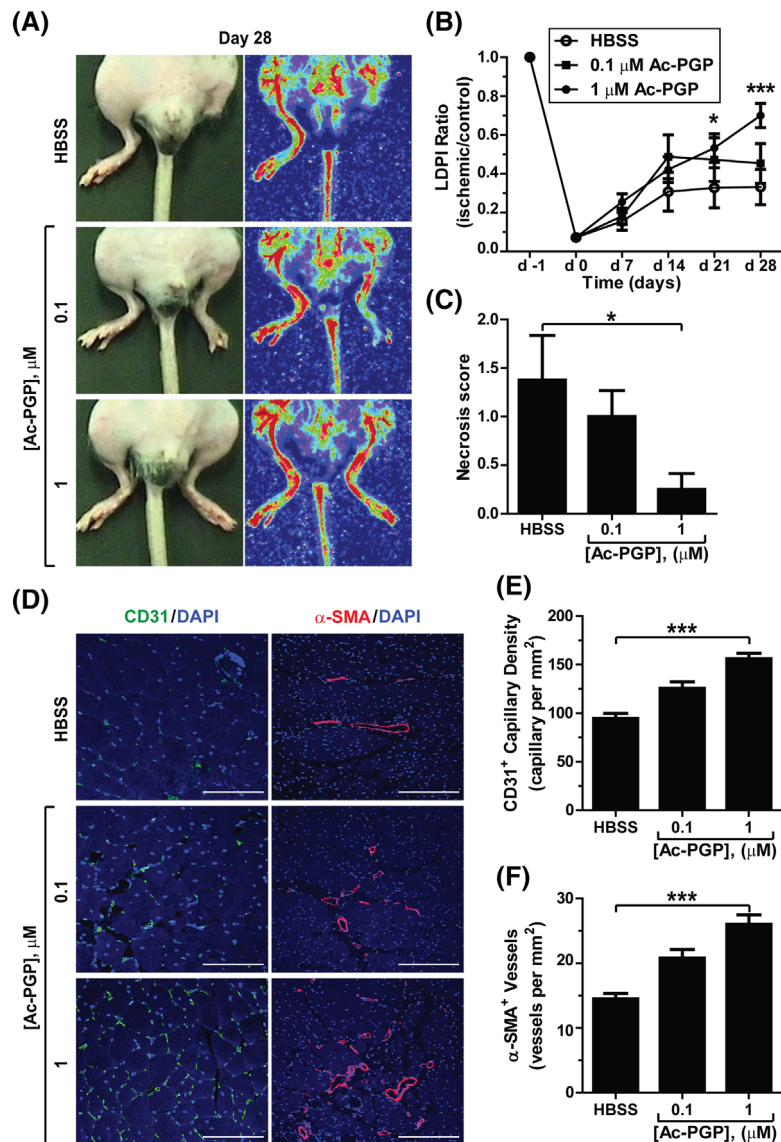


Figure 1. Effects of N-acetylated proline-glycine-proline (Ac-PGP) on blood perfusion, necrosis, and neovascularization in ischemic murine hind limbs. **(A):** Representative photographs and laser Doppler perfusion imaging (LDPI) images of mouse hind limbs injected intramuscularly with the indicated concentrations of Ac-PGP for 28 days. **(B):** Quantitative analysis of the blood perfusion recovery as determined by LDPI. LDPI ratio was defined as the ratio of ischemic to nonischemic hind limb blood perfusion. Results are presented as mean \pm SD ($n = 8$). **(C):** Statistical analysis of necrosis scores obtained 28 days after femoral artery excision and the commencement of Ac-PGP administration. Results are presented as mean \pm SD ($n = 8$). **(D):** Immunostaining of CD31-positive capillaries (green color) or α -SMA-positive blood vessels (red color) in ischemic limbs treated intramuscularly with 0, 0.1, or 1 μ M Ac-PGP at 28 days after surgery. Nuclei (blue color) were counterstained with DAPI; overlaid images are shown. Bar = 100 μ m (CD31/DAPI) or 200 μ m (α -SMA/DAPI). **(E):** Quantitative analysis of CD31-positive capillaries in ischemic limbs as determined by immunohistochemistry. Results are presented as mean \pm SD ($n = 8$). **(F):** Quantitative analysis of α -SMA-positive blood vessels in ischemic limbs as determined by immunohistochemistry. Results are presented as mean \pm SD ($n = 24$). *, $p < .05$; ***, $p < .001$.

Ac-PGP on angiogenesis in vivo, we used an immunostaining technique to assess blood vessel densities in ischemic limbs. CD31-positive capillary densities were found to be greater in ischemic limbs injected with 1 μ M Ac-PGP than in control limbs (Fig. 1D, 1E). Similarly, the densities of α -SMA-positive arteries/arterioles were also greater in ischemic limbs treated with 1 μ M Ac-PGP than in HBSS treated controls (Fig. 1D, 1F). Furthermore, these findings of increased densities of CD31-positive capillaries and α -SMA-positive arterioles/arteries after Ac-PGP injection were consistent with the increased blood perfusion and reduced necrosis severities observed in Ac-PGP

treated limbs. Overall, these results indicate Ac-PGP administration increased blood perfusion and reduced necrosis by stimulating angiogenesis.

CXCR2 was Required for the Protection and Promotion of Neovascularization in Ischemic Tissues by Ac-PGP

To clarify the role played by CXCR2 in Ac-PGP-induced ischemic tissue repair and angiogenesis in vivo, we applied our ischemic limb model to CXCR2 knockout mice. Intramuscular administration of Ac-PGP significantly improved blood perfusion as compared with HBSS-injected control groups in the

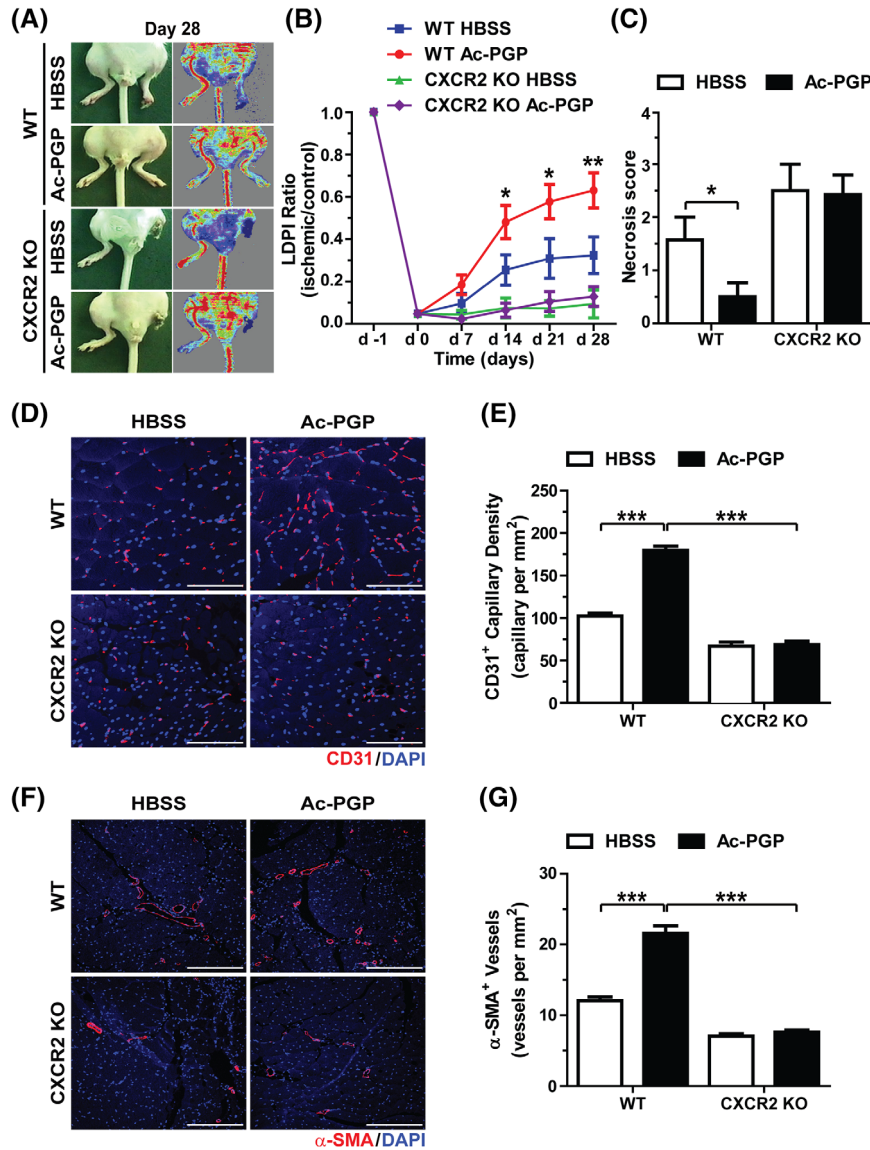


Figure 2. Role played by CXCR2 in N-acetylated proline-glycine-proline (Ac-PGP)-induced functional recovery and neovascularization in ischemic hind limbs. **(A)**: Representative photographs and laser Doppler perfusion imaging (LDPI) images of the hind limbs of wild-type (WT) or CXCR2-knockout (CXCR2 KO) mice intramuscularly administered with HBSS or Ac-PGP (1 μ M) at 28 days after surgery. **(B)**: Quantitative analysis of blood perfusion recovery as determined by LDPI. LDPI ratio was defined as the ratio of ischemic to nonischemic hind limb blood perfusion. Results are presented as mean \pm SD. *, $p < .05$; **, $p < .01$ for WT Ac-PGP versus WT Hank's balanced salt solution mice ($n = 9$). **(C)**: Necrosis score results on day 28. Results are presented as mean \pm SD ($n = 8$). **(D)**: Representative images of mouse ischemic limbs obtained 28 days after surgery and immunostained with anti-CD31 antibody (red color). Nuclei were counterstained with DAPI (blue color). Bar = 100 μ m. **(E)**: Quantitative analysis of CD31-positive capillaries in ischemic limbs by immunohistochemistry. Results are presented as mean \pm SD ($n = 9$). **(F)**: Representative images of mouse ischemic limbs at days 28 after surgery and immunostaining with anti- α -SMA antibody (red color). Nuclei were counterstained with DAPI (blue color). Bar = 200 μ m. **(G)**: Quantitative analysis of α -SMA-positive vessels in ischemic limbs by immunohistochemistry. Results are presented as mean \pm SD. *, $p < .05$; ***, $p < .001$ ($n = 9$).

ischemic limbs of wild-type mice (Fig. 2A, 2B). On the other hand, CXCR2 KO mice exhibited substantially impaired blood perfusion recovery at day 28 after femoral artery excision as compared with wild-type mice (Fig. 2A, 2B), and Ac-PGP treatment failed to improve blood perfusion in ischemic limbs. Furthermore, intramuscular administration of Ac-PGP resulted in a time-dependent increase in LDPI ratio in wild-type mice, but not in CXCR2 KO mice. These results suggested the involvement of CXCR2 in the blood perfusion improvement induced by Ac-PGP. In CXCR2 KO mice, intramuscular administration of Ac-PGP did not significantly reduce tissue necrosis

or amputation rates of ischemic limbs (Fig. 2A, 2C). To determine whether CXCR2 was involved in Ac-PGP-induced angiogenesis in ischemic limbs, we investigated blood vessel densities in the ischemic limb tissues of wild-type and CXCR2 KO mice. Intramuscular administration of Ac-PGP significantly increased numbers of CD31-positive capillaries in wild-type mice at day 28 after surgery (Fig. 2D, 2E). However, in CXCR2 knockout mice, Ac-PGP treatment did not significantly increase numbers of CD31-positive capillaries in ischemic limbs. In addition, Ac-PGP increased α -SMA-positive blood vessel densities in wild-type mice, but not in CXCR2 KO mice (Fig. 2F, 2G).

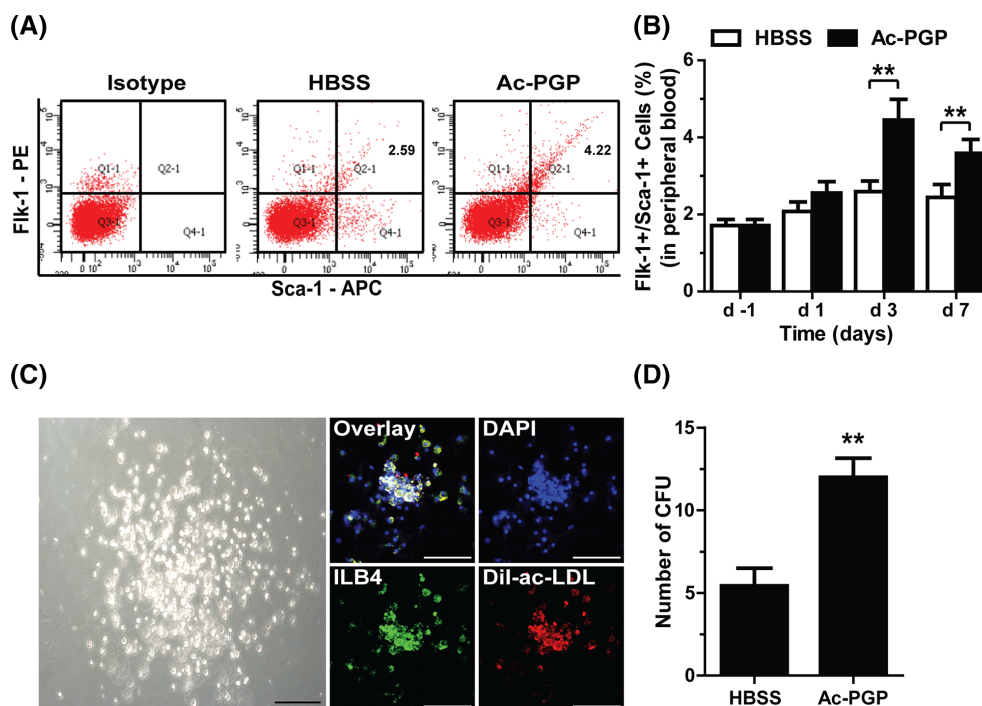


Figure 3. Effect of intramuscular N-acetylated proline-glycine-proline (Ac-PGP) injections on circulating angiogenic cell (CAC) mobilization into peripheral blood. **(A):** Representative flow cytometry plots and percentages of Flk-1⁺/Sca-1⁺ mononuclear cells (MNCs) in PB 3 days after injecting ischemic wild-type mice with Hank's balanced salt solution (HBSS) or 1 μ M Ac-PGP in HBSS. **(B):** Time-responses of CAC mobilizations to Ac-PGP. Percentages of Flk-1⁺/Sca-1⁺ MNCs in PB were determined at the indicated time after injecting 1 μ M Ac-PGP into ischemic hind limbs. Results are presented as mean \pm SD ($n = 17$). **(C):** Representative images of colonies obtained by culturing PB MNCs from HBSS- or Ac-PGP (1 μ M)-injected mice (left panel). Colonies were identified by immunostaining with DAPI (blue color), fluorescein ILB4 (green color), and Dil-ac-LDL (red color; light panel) after culture for 12 days. Bar = 100 μ m. **(D):** Colony forming units produced from PB MNCs during the colony-forming assay. Results are presented as mean \pm SD ($n = 7$). **, $p < .01$.

These results suggest CXCR2 played a principal role in the observed blood perfusion and neovascularization improvements observed in ischemic limbs.

Ac-PGP Stimulated the Mobilization of CACs into Peripheral Blood

Ischemia has been reported to induce the mobilization of CACs from bone marrow to the circulation [32, 33], and these cells are known to play a key roles in the regeneration of blood vessels and repair of ischemic tissues [13, 14]. When Ac-PGP was administered daily to the ischemic limbs of wild-type C57BL/6J mice, percentages of Flk-1⁺/Sca-1⁺ CACs in PB increased time-dependently and peaked on day 3 (Fig. 3A, 3B). The population of CD31-positive cells in the Flk-1⁺/Sca-1⁺ CACs was increased in Ac-PGP-treated mice, in contrast to the decrease of CD45-positive cells in Flk-1⁺/Sca-1⁺ CACs (Supporting Information Fig. S1). To confirm these results, we examined the effect of Ac-PGP on numbers of colony-forming cells in PB. PB MNCs isolated from HBSS- or Ac-PGP-administered mice were subjected to a colony-forming assay. It was found that the colonies exhibited positivity for ILB4- and Dil-ac-LDL (Fig. 3C), and more colonies were produced by the PB MNCs of Ac-PGP-administered mice than by those of HBSS-administered mice (Fig. 3D). The Dil-ac-LDL-positive colonies highly expressed CD31, an endothelial marker, and a few CD45-positive leukocytes were detected in the colonies (Supporting Information Fig. S2). These results suggest Ac-PGP promoted the mobilization of endothelial colony-forming CACs into PB.

CXCR2 was Required for the Ac-PGP-Induced Mobilization of CACs into Peripheral Blood

To evaluate the role played by CXCR2 in the Ac-PGP-induced mobilization of CACs into PB, we compared the effects of Ac-PGP on CAC mobilization in wild-type and CXCR2 KO mice. When Ac-PGP or HBSS were intramuscularly injected daily into the ischemic limbs of wild-type mice for 3 days, Ac-PGP increased Flk-1⁺/Sca-1⁺ CAC mobilization; however, this effect was not observed in CXCR2 KO mice (Fig. 4A, 4B). Moreover, intramuscular administration of Ac-PGP to wild-type mice increased numbers of colony forming cells in PB more so than in HBSS-treated controls, and this was also not observed in CXCR2 KO mice (Fig. 4C). These results suggest that CXCR2 activation plays a crucial role in the Ac-PGP-induced mobilization of colony-forming CACs.

Ac-PGP Accelerated the Neovascularization and Recruitment of Bone Marrow-Derived CACs in the Hind Limb Ischemia Model

Bone marrow cells isolated from green fluorescent protein (GFP) transgenic mice were transplanted into irradiated wild-type mice, which were then subjected to hind limb ischemia and intramuscularly injected with Ac-PGP (Fig. 5A). Ac-PGP treatment increased the population of GFP⁺/Flk-1⁺/Sca-1⁺ CACs in PB (Supporting Information Fig. S3). In the ischemic limbs of bone marrow-recipient mice, Ac-PGP was observed to time-dependently increase blood perfusion (Fig. 5B, 5C).

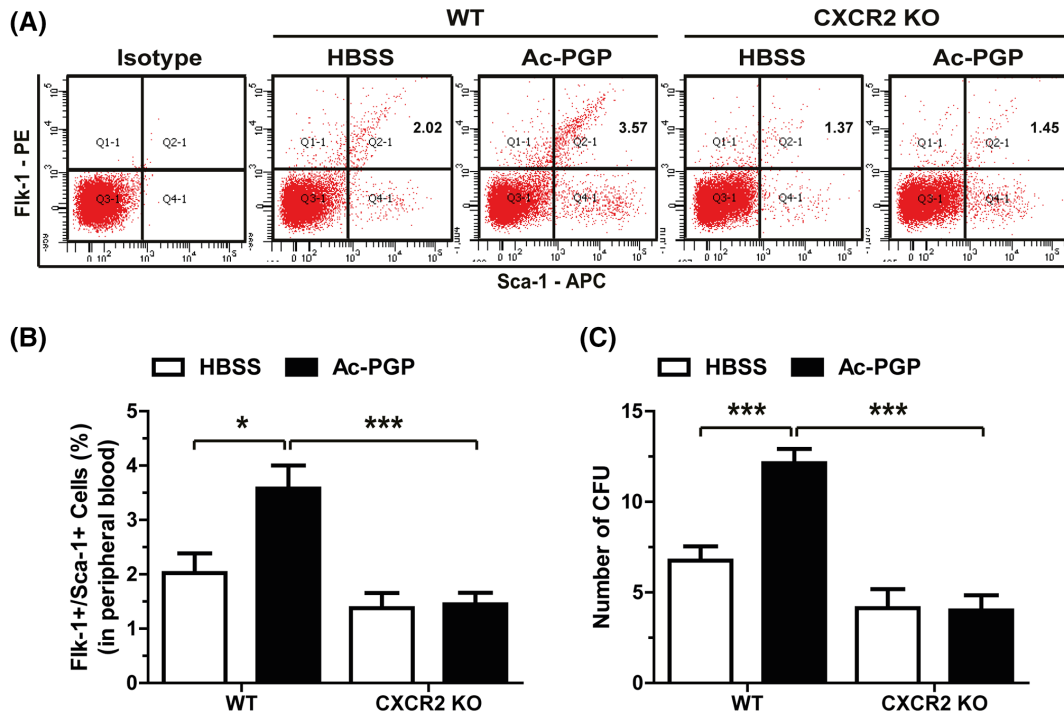


Figure 4. Role played by CXCR2 in the N-acetylated proline-glycine-proline (Ac-PGP)-induced mobilization of circulating angiogenic cells (CACs) into peripheral blood. **(A):** Representative flow cytometry plots and percentages of FLK-1⁺/Sca-1⁺ mononuclear cells (MNCs) in PB after daily injecting ischemic limbs of wild-type or CXCR2 knockout (KO) mice with Hank's balanced salt solution (HBSS) or Ac-PGP (1 μ M) for 3 days. **(B):** CAC mobilizations induced by HBSS or HBSS containing Ac-PGP (1 μ M). Results are presented as mean \pm SD ($n = 12$). **(C):** Quantitative analysis of colony-forming units from PB MNCs derived from wild-type or CXCR2 KO mice injected daily with HBSS or HBSS containing Ac-PGP (1 μ M) for 3 days, as determined by a colony-forming assay. Results are presented as mean \pm SD ($n = 8$). *, $p < .05$; ***, $p < .001$.

Furthermore, when ischemic limb tissues obtained at 28 days after surgery were stained with ILB4 (specifically labels blood vessels), Ac-PGP-treated ischemic limbs were found to have more ILB4-positive capillaries than control tissues (Fig. 5D, 5E), and greater numbers of α -SMA-positive vessels (Fig. 5D, 5F).

GFP-positive bone marrow-derived cells were also detected in the ischemic limbs of wild-type recipient mice transplanted with bone marrow from GFP-transgenic mice, and numbers of GFP-positive cells in Ac-PGP-injected ischemic limbs were greater than in control limbs (Fig. 6A, 6B), suggesting Ac-PGP administration facilitated the recruitment of bone marrow-derived cells. Next, we investigated the effect of Ac-PGP injection on bone marrow-derived EC-mediated neovascularization by counting numbers of capillaries and vessels doubly positive for GFP positive cells and either ILB4 or α -SMA, respectively. Substantially greater GFP- and ILB4-double positive capillary densities were observed in the ischemic limb tissues of Ac-PGP treated mice than in HBSS treated control ischemic mice (Fig. 6C, 6D), and the ischemic muscle tissues of Ac-PGP treated mice also contained greater numbers GFP- and α -SMA-double positive vessels (Fig. 6E, 6F). These results suggest that Ac-PGP induced the recruitment of bone marrow-derived ECs into ischemic tissues and promoted neovascularization after ischemia.

DISCUSSION

Angiogenesis is important for recovery from ischemic diseases, such as, myocardial infarction, cerebral stroke, and peripheral

arterial disease [31, 34]. In the present study, we demonstrated that Ac-PGP administration enhanced blood perfusion and attenuated tissue necrosis in a murine hind limb model of ischemia. In addition, our results indicate a CXCR2-dependent mechanism was responsible for the observed Ac-PGP-induced increases in capillary and arteriole/artery numbers, thus suggesting CXCR2 plays a pivotal role in Ac-PGP-mediated ischemic tissue repair and angiogenesis.

CXCR2 is the main receptor involved in neutrophil, monocyte, and macrophage chemotaxis and is known to play important functional roles in host defense, inflammation, and angiogenesis [35, 36], as exemplified by reports of larger chronic lesion sizes, poorer functional outcomes, and impaired angiogenesis in CXCR2 knockout mice [37, 38]. CXCR2 has also been reported to be involved in the regulation of the angiogenic capacities of endothelial cells by IL-8 [36], and in another study, blockade of CXCR2 expression in endothelial cells attenuated IL-8-induced angiogenic responses [39]. Ac-PGP increased the tube-forming activity of human umbilical vein endothelial cells in vitro and blockade of CXCR2 by SB225002, a CXCR2 antagonist, abrogated the Ac-PGP-induced endothelial tube formation (Supporting Information Fig. S4), suggesting a key role of CXCR2 in the Ac-PGP-induced angiogenesis. Furthermore, Ac-PGP, which has high affinity for CXCR2, was found to stimulate polymorphonuclear cells and to be a player in various diseases, including pulmonary and inflammatory diseases [40, 41]. These findings suggest CXCR2 plays pivotal roles in the Ac-PGP-induced repair of ischemic tissues and in Ac-PGP-induced angiogenesis.

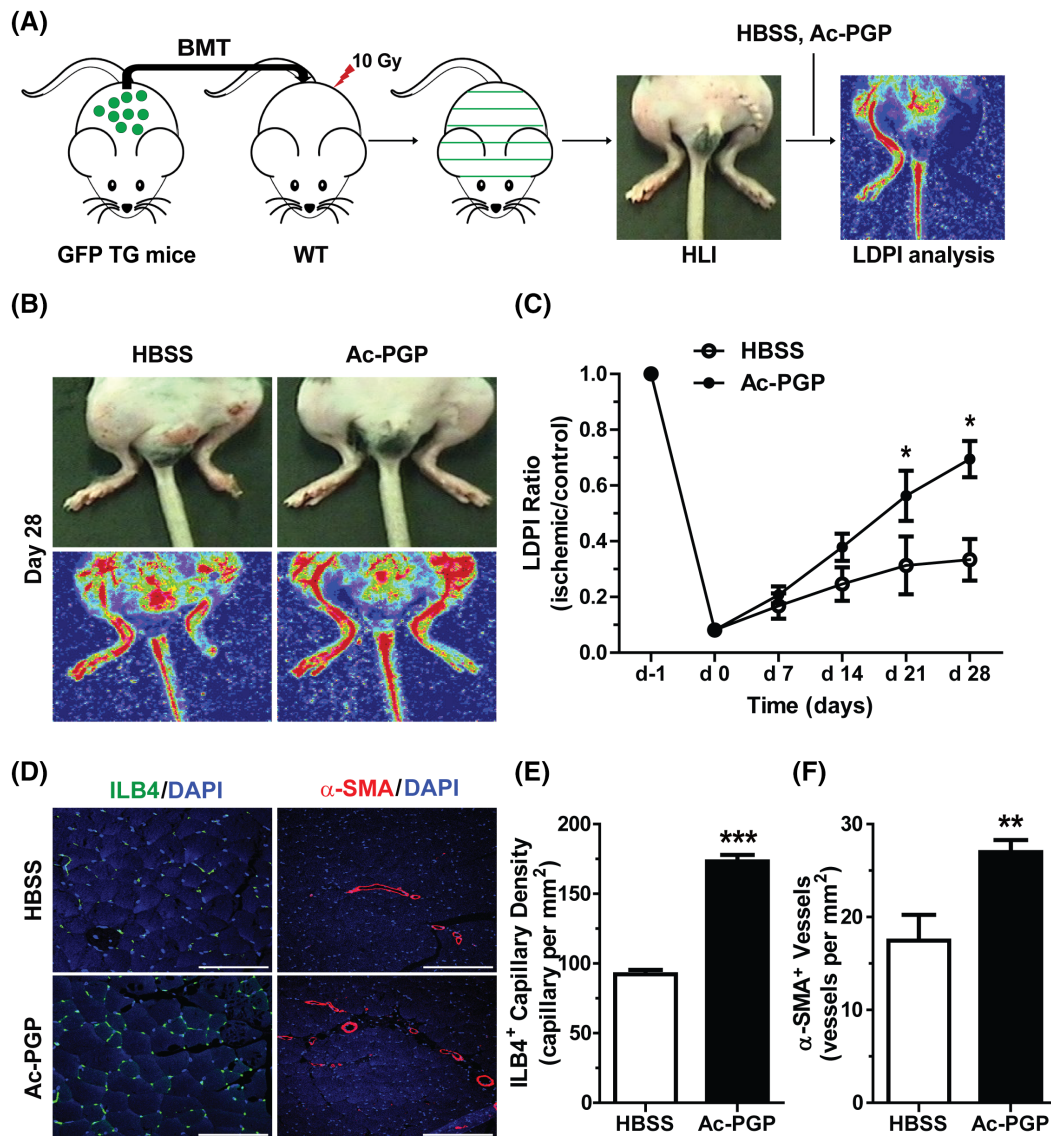


Figure 5. Effects of N-acetylated proline-glycine-proline (Ac-PGP) on neovascularization in the ischemic hind limbs of bone marrow-transplanted mice. **(A):** Green fluorescent protein (GFP) transgenic mouse (C57BL/6-Tg[CAG-EGFP]10sb/J)-derived bone marrow cells (GFP⁺ bone marrow cells) were transplanted into irradiated wild-type mice, subjected to femoral artery excision, and injected with Hank's balanced salt solution (HBSS) or HBSS containing Ac-PGP (1 μ M) intramuscularly into ischemic limbs. **(B):** Representative photographs and laser Doppler perfusion imaging (LDPI) images of bone marrow transplanted recipient mice injected with HBSS or HBSS containing Ac-PGP (1 μ M) on day 28 after inducing hind limb ischemia. **(C):** Blood perfusion recovery as determined by LDPI. LDPI ratios were calculated by dividing ischemic hind limb perfusion by nonischemic hind limb perfusion. Results are presented as mean \pm SD ($n = 7$). **(D):** Fluorescence images of ILB4 (green color) and α -SMA-positive blood vessels (red color) in ischemic limb samples (obtained 28 days after surgery) of mice injected with HBSS or HBSS containing Ac-PGP (1 μ M). Nuclei (blue color) were counterstained with DAPI; overlaid images are shown. Bar = 100 μ m (ILB4/DAPI) or 200 μ m (α -SMA/DAPI). **(E):** Numbers of ILB4-positive capillaries in ischemic limbs were quantified from the data shown in panel (D). Results are presented as mean \pm SD ($n = 7$). **(F):** The numbers of α -SMA-positive vessels in ischemic hind limbs were quantified from the data shown in panel (D). Results are presented as mean \pm SD ($n = 7$). *, $p < .05$; **, $p < .01$; ***, $p < .001$.

It has been suggested CACs are recruited to injured tissues to accelerate neovascularization and injured tissue repair [42], and that the mobilization of CACs from bone marrow into peripheral blood is required for therapeutic angiogenesis, which leads to the incorporation of CACs into ischemic tissues and enhanced neovascularization [13, 14]. The mobilization of CACs from bone marrow to peripheral blood is induced by a variety of stimuli, which include G-CSF, stromal-derived factor-1, VEGF, and IL-8/GRO- β [43]. In an *in vitro* study, IL-8 was observed to enhance the angiogenic activities of umbilical cord

blood-derived endothelial progenitor cells [44], and *in vivo*, CXCR2 was found to be involved in the homing of CACs to sites of arterial injury and in endothelial recovery [45]. In addition, bone marrow-derived CAC mobilization was reported to be impaired in CXCR2-knockout mice harboring pancreatic cancer [46]. In the present study, Ac-PGP administration induced CAC mobilization in wild-type mice, but did not induce CAC mobilization in CXCR2 KO mice. Consistently, the number of CXCR2-positive cells was increased in Ac-PGP-treated limbs (Supporting Information Fig. S5), suggesting mobilization of

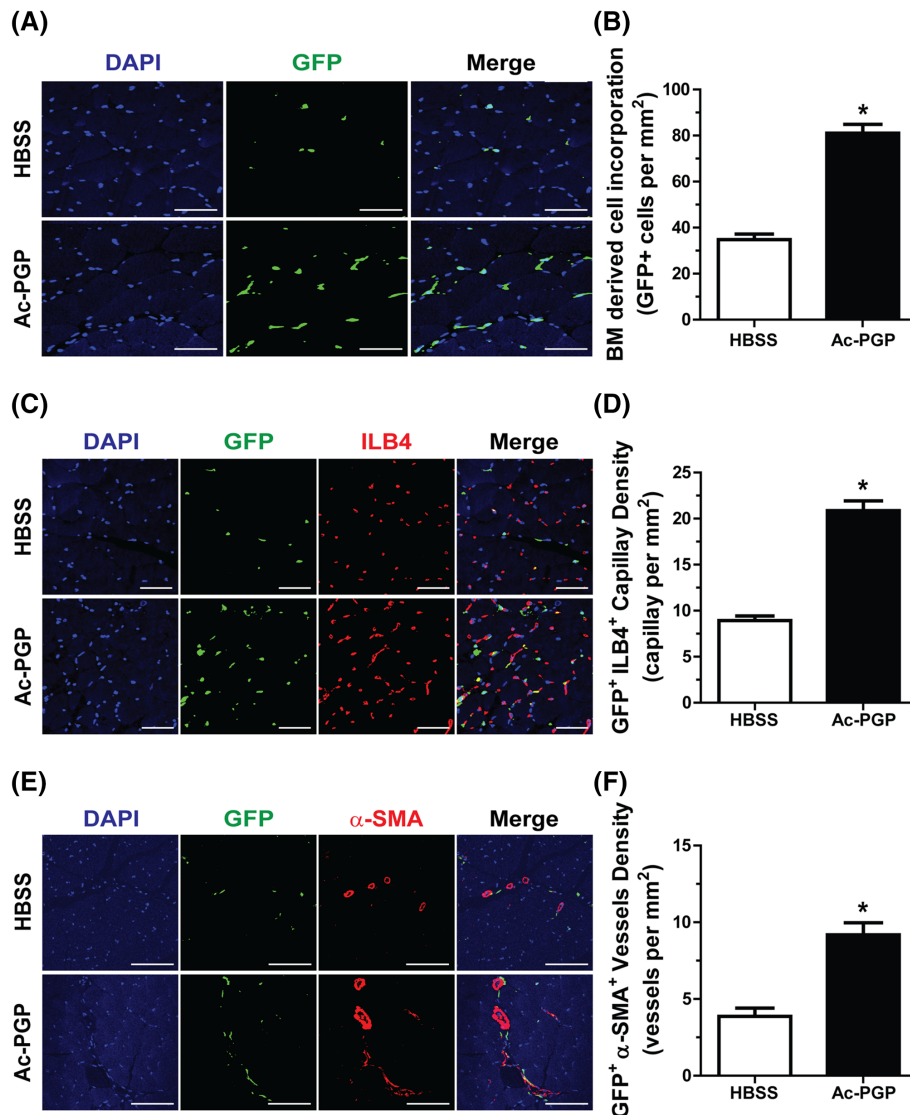


Figure 6. Effects of N-acetylated proline-glycine-proline on bone marrow-derived cell neovascularization in ischemic hind limbs. **(A):** Representative images of ischemic hind limbs obtained 28 days after femoral artery excision and staining with anti-GFP antibody (green color). Nuclei were counterstained with DAPI (blue color). Bar = 50 μ m. **(B):** Numbers of green fluorescent protein (GFP)-positive cells in ischemic limbs were quantified from the data shown in panel (A). **(C):** Representative images of hind limb tissues doubly stained with anti-GFP (green color) and anti-ILB4 (red color) antibody at 28 days after surgery. Nuclei were counterstained with DAPI (blue color). Bar = 50 μ m. **(D):** Numbers of GFP- and ILB4-double positive capillary in ischemic limbs were quantified from the data shown in panel (C). **(E):** Representative images of ischemic hind limb tissues after double staining with anti-GFP (green color) and anti- α -SMA (red color) antibody. Nuclei were counterstained with DAPI (blue color). Bar = 100 μ m. **(F):** Numbers of GFP- and α -SMA-double positive vessels in ischemic limb muscles were quantified from the data shown in panel (E). Results are presented as mean \pm SD ($n = 9$). *, $p < .05$.

CXCR2-positive cells. It has been previously reported, the CXCR2 ligands, IL-8 and GRO- β , mobilized CACs by activating MMP-9, causing the release of soluble Kit ligand and the subsequent mobilization of bone marrow endothelial progenitor cells [47, 48], and that IL-8 and GRO- β appear to work synergistically with G-CSF to mobilize stem and progenitor cells in bone marrow [49, 50]. Ac-PGP treatment did not affect the levels of CXCL2, a murine homolog of IL-8, in PB (Supporting Information Fig. S6), indicating that Ac-PGP treatment directly induces CAC mobilization, but not by elevating CXCL2 levels in PB. These results suggest Ac-PGP might be valuable for treating peripheral artery diseases by mobilizing CACs to peripheral tissues.

In the present study, the mobilization of Flk-1⁺/Sca-1⁺ CACs in peripheral blood was increased by intramuscular injections of Ac-PGP and peaked 3 days after excision of hind limb femoral artery. The Flk-1⁺/Sca-1⁺ CACs expressed not only the endothelial marker CD31 but also the leukocyte maker CD45, suggesting heterogeneity of CACs. However, Ac-PGP treatment increased the population of CD31-positive cells, but not CD45-positive cells in the Flk-1⁺/Sca-1⁺ CACs. In addition, in bone marrow-reconstituted mice, Ac-PGP stimulated the recruitment of bone marrow-derived CACs to ischemic tissues and promoted incorporation of bone marrow-derived CACs into newly generated ILB4- and α -SMA-positive blood vessels.

Flk-1⁺/Sca-1⁺ cells help protect ischemic muscles by enhancing angiogenesis in hind limb ischemia [51], and have been shown to reduce mortality and cardiac dysfunction after myocardial infarction [52]. We recently reported the activation of formyl peptide receptor 2 led to the mobilization of Flk-1⁺/Sca-1⁺ CACs from bone marrow and to subsequent repair of infarcted myocardium. Furthermore, Flk-1⁺/Sca-1⁺ cells isolated from bone marrow of wild-type mice were found to recapitulate the therapeutic effects of formyl peptide receptor activation [53, 54]. These reports support our finding that Ac-PGP accelerates the mobilization of Flk-1⁺/Sca-1⁺ CACs, and thus, neovascularization in ischemic tissues. However, we observed that all of newly formed blood vessels are not positive for bone marrow-derived GFP-positive cells, therefore, it cannot be excluded that tissue-resident endothelial progenitor/stem cells contribute in the angiogenesis and tissue repair. Accumulating evidence suggests the presence of tissue-resident endothelial progenitor/stem cells and their contribution in tissue regeneration [55, 56]. However, the source of stem cells in the revascularization is still ambiguous, therefore, the contribution of bone marrow-derived CACs and tissue-resident stem cells in the revascularization needs to be clarified further.

CONCLUSION

In the present study, Ac-PGP administration stimulated the recruitment of bone marrow-derived CACs to ischemic limb

tissues. This localization of bone marrow-derived CACs in ischemic tissues offers a means of promoting angiogenesis and ischemic tissue repair, and thus, the possibility of a novel, efficient, therapeutic strategy [51]. Taken together, our findings suggest that the Ac-PGP-CXCR2 axis offers a unique opportunity to develop a CAC-mediated therapy for ischemia.

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AUTHOR CONTRIBUTIONS

Y.K. and S.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.H., T.L., G.P., J.Y., S.K., H.S.: collection and/or assembly of data; S.L.: conception and design, supervision of the study; J.K.: conception and design, supervision of the study, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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