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G-CSF Administration Accelerates Cutaneous Wound Healing Accompanied With Increased Pro-Hyp Production In *db/db* Mice

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Abstract

Objective: Impaired wound healing in diabetic patients is a clinical concern. However, exacerbation factors in diabetic wounds are still not clear. Inflammatory cell infiltrates after skin wounding and subsequent healing responses were investigated using diabetic mice.

Methods: Granulocyte colony-stimulating factor (G-CSF), neutrophil infiltration and peptides from degraded collagen in wounded tissue were examined using db/db and wild-type mice.

Results: The collagen peptides Pro-Hyp and Hyp-Gly in wounded tissue were quantified. G-CSF was transiently secreted from wounded tissue immediately after excision and then appeared in peripheral blood. G-CSF levels were significantly lower in db/db mice than in wild-type mice, and neutrophil infiltration into the granulation tissue was lower in db/db mice. In wound tissue, only Pro-Hyp increased during the 7-day study, and Pro-Hyp levels were significantly lower in db/db mice. Wound closure was severely impaired in db/db mice. However, topical recombinant human G-CSF (rhG-CSF) administration accelerated healing, accompanied with increased neutrophil infiltration and Pro-Hyp production.

Conclusion: The results show that decreased G-CSF secretion in wound tissue may trigger delayed healing in diabetic mice and that topical rhG-CSF administration increased Pro-Hyp production and accelerated healing. Therefore, G-CSF-induced Pro-Hyp may play an important role in wound healing.

Keywords: Collagen; Diabetes; G-CSF, Mice; Neutrophil; Pro-Hyp; Wound healing

Abbreviations

G-CSF: Granulocyte Colony-Stimulating Factor; rhG-CSF: Recombinant Human G-CSF; Pro-Hyp: Proline-Hydroxyproline; Hyp-Gly: Hydroxyproline-Glycine; ECM: Extracellular Matrix;

Introduction

With the increasing number of diabetic patients, treatment for delayed healing of wounds, including foot diseases and ulcerations, has become a serious medical concern worldwide [1]. Many approaches to treatment have been developed; Negative-pressure wound therapy is one option to remove the harmful excess fluid that results from delayed wound healing and improve blood circulation, leading to more favorable outcomes [2]. It has also been shown that growth factor therapy using basic fibroblast growth factor promotes wound healing through its positive effects on fibroblast and keratinocyte growth and neovascularization [3]. However, the available strategies to address non-healing wounds that arise from various pathologies are still limited. Therefore, it is necessary to explore the novel tissue factors and/or mechanisms involved in delayed wound healing.

An inflammatory cascade takes place after wounding, and phagocytic cells, including neutrophils, are recruited from the bloodstream [4-6]. The density of inflammatory cells and/or the duration of inflammation are important factors that influence wound healing. Prolonged production of proinflammatory cytokines, reactive oxygen species, and protein-digestive enzymes can give rise to uncontrolled leukocyte accumulation. This pathological state can be observed in the chronic refractory wounds that accompany peripheral vascular disease and diabetes [7]. Wound healing is also delayed in a neutropenic state [8]. Thus, there is a delicate balance between healing and inflammation in healing wounds.

The mechanism of inflammatory cell infiltration following injury has been thoroughly investigated [4]. When an injury occurs, neutrophils are immediately mobilized from the bone marrow and other storage organs. Neutrophils stimulated by

IL-8 and leukotriene from the wounded tissue, recognize selectin expressed on endothelial cells and start rolling, and then attach to intercellular adhesion molecule-1. Neutrophils then penetrate the endothelial monolayer through recognition of platelet/endothelial cell adhesion molecule-1 and transmigrate into the tissue matrix. Finally, the neutrophils reach the wound via the gradient of migration factors.

Neutrophils are produced in the bone marrow following stimulation with proinflammatory cytokines, stem cell factor (SCF), and granulocyte colony-stimulating factor (G-CSF), which induce their differentiation, and mature neutrophils are mobilized into the bloodstream [9]. G-CSF is used clinically to treat neutropenic patients and to harvest hematopoietic stem cells. However, it is also known that G-CSF possesses a variety of functions, such as, endothelial-progenitor cell mobilization [10], stimulation of endothelial proliferation [11], mesenchymal stem cell mobilization [12], and augmentation of antibacterial function [13]. Since all these activities favor wound healing, many clinical studies of G-CSF have been conducted on refractory wounds in neutropenic patients [14], diabetic foot infections [15], and acute myocardial infarctions [16].

The sequential steps in wound healing are inflammation, granulation tissue formation, wound contraction, and epithelial regeneration [6, 17, 18]. These steps proceed in a serial and overlapping manner. Of these steps, the formation of granulation tissue is critical because it acts as a scaffold for epidermal regeneration and an axis for the blood supply needed to maintain cellular activities [19]. The basic structure of granulation tissue consists of fibroblasts and extra cellular matrix (ECM) components, including collagens, which act as a scaffold for endothelial cells to form neovascular networks [20]. In the final step, a wound is covered by regenerative epithelial cells, and the granulation tissue regresses.

An important function of neutrophils is ECM degradation, which leads to the remodeling of healing tissue [18]. In studies on mice that overexpress the collagen catabolic enzyme matrix-metalloproteinase-8 (MMP-8) [21] and MMP-8 knockout mice [21], wound healing is delayed. It has also been shown that metabolites of collagen possess biological functions. Ingested collagens are supposed to be digested to single amino acids. However, collagen dipeptides, including proline-hydroxyproline (Pro-Hyp) and hydroxyproline-glycine (Hyp-Gly), appear for a certain time in the blood [22]. It has been shown that these dipeptides exert biological effects on skin barrier function [23] and fibroblast growth [24].

In the present study, with an aim to clarify the effect of G-CSF administration on refractory diabetic wounds, we examined the relationship between neutrophil infiltration and collagen-derived peptides in wounds using genetically diabetic db/db mice. This is the first report to clearly show that the collagen-derived dipeptide Pro-Hyp is produced in inflamed wound tissue, which may play a role in wound healing.

Methods

Animals and wounds

Male db/db (C57BLKS/Jlar-+Leprdb/+Leprdb) and wild-type (C57BLKS/Jlar- m+Leprdb/m+Leprdb) 10–15-week-old mice and male C57BL/6 8–10-week-old mice were used in this study. All animals were purchased from Japan SLC Inc. (Shizuoka, Japan). Hematological analyses, including red blood cell (RBC), white blood cell (WBC), and platelet (PLT) counts (Celltac-α, NIHON KODEN, Tokyo, Japan) and blood glucose concentration (Glutest Sensor; Sanwa Chemical, Aichi, Japan), were performed with blood collected from the orbital sinus with a heparinized 75-μL capillary (Hirschmann Laborgerate, Germany). At the end of study, the mice were sacrificed by lethal pentobarbital injection and arterial hemorrhage.

All procedures were conducted under aseptic conditions, using autoclaves, ethylene oxide gas, 70% ethanol, and povidone-iodine. Mice were anesthetized with pentobarbital (Somnopentyl; KYORITU SEIYAKU, Tokyo, Japan). After removing the dorsal hair with a commercial depilatory, a circular tattoo (1 cm in diameter) was made at the center of the lumbar area, and this section of skin was completely excised with scissors. Finally, the wound was covered with a polyurethane film dressing (Tegaderm; SUMITOMO 3M, Tokyo, Japan).

Measurement of G-CSF

G-CSF was measured in blood and tissue samples. Blood samples were obtained from the orbital sinus 3–96 hours after wounding. Tissue samples were also obtained from the wounded and normal subcutaneous tissue on the back of the same mice 3–24 hours after wounding. After weighing the tissue, it was kept in ice-cold protein extraction buffer (RIPA Lysis Buffer; MERCCK MILLIPORE Co., Darmstadt, Germany). The tissue was then homogenized with a Biomasher (Nippi Inc., Tokyo, Japan). After centrifugation at 9000 g for 15 min at 4°C, the supernatant was used for measurement. The G-CSF concentration in blood and tissue was determined with the mouse G-CSF immunoassay kit (R&D Systems Inc., MN, USA), and the absorbance was measured with a microplate reader (SUNRISE Rainbow RC-R; TECAN Japan, Kawasaki, Japan) at 450 nm/540 nm.

Measurement of collagen peptides

Wounded and normal subcutaneous tissue samples (1 cm in diameter) were collected at 1, 3, 5, and 7 days after skin excision and weighed. Samples were immersed in 75% ethanol and stored in a deep freezer until analysis. Tissue was homogenized in a Biomasher and centrifuged at 9,000 g for 15 min at 4°C. The supernatant was used to measure Hyp-Gly and Pro-Hyp levels. Peptides in the samples were derivatized with 6-aminoquinolyl-N-hydroxy succinimidyl carbamate using the AccQ Tag kit (Waters, Milford, MA, USA). The derivatives were resolved by reversed phase HPLC using an Inertsil ODS-3 column (2 mm × 250 mm; GL Sciences, Tokyo, Japan) with a binary gradient elution consisting of 0.1% formic acid and 80% acetonitrile containing 0.1% formic acid and detected by MS/MS using a 3200 QTRAP (SCIEX, Framingham, MA, USA) equipped with an electron spray

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ionization probe in multi-reaction monitoring (MRM) mode. Optimization of the MRM conditions and quantification of the peptides were performed using installed software, Analyst and MultuQuant (AB CIEX, MA, USA), respectively.

Administration of recombinant human G-CSF

After skin excision, the subcutaneous splint model [25] was used to mimic human wound healing. The wound was sealed with a polyurethane film dressing, and 25 μg/100 μL recombinant human G-CSF (rhG-CSF: filgrastim; Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) or vehicle alone was injected onto the wound surface. On days 0, 3, and 5, the wounds were photographed along with a scale. The wound area was measured using a computer-assisted morphometric analyzer (VH Analyzer VH-H1A5; KEYENCE Co., Osaka, Japan). On days 3 and 5, the mice were sacrificed. Wounded tissue, including the splint, was dissected, and used for histological examination.

Histological examination

The wound tissue was fixed in 10% buffered formaldehyde (pH 7.4) for several days. Two cross-cut tissue samples from each wound were excised and embedded in paraffin blocks using a tissue processor (Tissue-Tec VIP Premiere; Sakura Seiki Co. Ltd., Nagano, Japan). Then, 4-μm-thick tissue sections were cut with a microtome (RM2235; Leica Biosystems, Nußloch, Germany). For frozen specimens, another tissue sample was fixed in 10% buffered formaldehyde (pH 7.4) containing 10% sucrose for 24 hours, and then washed with 15% and 20% sucrose-containing buffer solutions (pH 7.4) for 24 hours. The tissue was immersed in tissue compound (OCT; Sakura Seiki Co. Ltd.), vacuumed for 30 min, and frozen in liquid nitrogen. The frozen tissue block was cut with a cryostat (CM3050S; Leica Biosystems) into 4-μm-thick sections.

Histological staining

Tissue sections were stained with Hematoxylin and Eosin (HE) and Masson's Trichrome (MT) stains. For immunohistological examination, the following primary antibodies were used: rabbit anti-mouse G-CSF antibody (1:200; Biorbyt Ltd., Kenbridge, UK), rat NIMP-R14 antibody (a Ly-6G/-6C neutrophil marker, 1:50; Hycult Biotech, Netherlands), and rabbit anti-MMP-8 antibody (1:200; Abcam plc, Tokyo, Japan) and diluted with Antibody Diluent (DAKO Japan Inc., Tokyo, Japan). For the G-CSF and MMP-8 antibodies, antigens were activated by heating in 0.01 M citrate buffer (pH 6.0) for 10 minutes. Sections were incubated with the primary antibody at 4°C overnight. The EnVision Kit (DAKO Japan Inc.) and a rat ABC staining system (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used for visualization. In double immunofluorescent staining, frozen sections were incubated with the NIMP-R14 and MMP-8 antibodies at room temperature for 2 hours in sequential order. After washing, sections were incubated with a cocktail of Alexa Fluor 488-labeled goat anti-rat IgG (1:2000; Thermo Fisher Scientific KK, Yokohama, Japan) and TRTIC-labeled goat anti-rabbit IgG (1:100; Proteintech Group Inc., Chicago, IL, USA). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich

Japan, Tokyo, Japan). Stained sections were observed under a microscope (Bio-Zero; KEYENCE Co., Osaka, Japan).

Evaluation of neutrophil infiltration and MMP-8 expression

Semi-quantitative evaluations of NIMP-R14-positive neutrophil infiltration and MMP-8 expression were performed using reference positive-stained photographs (0: absent, 1: several, 2: mild, 3: moderate, 4: severe, and 5: very severe). During the evaluations, the image assessment was blinded to the photographs that were used as evaluation criteria. Mean values were used.

Statistical analysis

Values were expressed as mean ± standard error. The two groups were compared using Student's t-test. P values less than 0.05 were considered significant.

Results

G-CSF production after skin excision

G-CSF production was evaluated in C57BL/6 mice. Basal levels of G-CSF in the blood and normal subcutaneous tissue without injury remained at minimum levels (Fig. 1A). However, 3 hours after skin excision, G-CSF levels in wounded tissue rapidly reached a maximum, and gradually decreased thereafter over the 24 hours of the study. However, no such increase was observed in the uninjured subcutaneous tissue on the same mice. Blood levels of G-CSF also increased and reached a maximum value 6 hours after skin excision, and then decreased over time. G-CSF-expressing cells were not detected in the exposed muscle fascia 1 hour after skin excision, but spindle- to oval-shaped cells were detected after 3 hours (Fig. 1B).

Physiological changes in db/db and wild-type mice after skin excision

Blood glucose and body weight of db/db mice were quite high, whereas those of wild-type mice were in the normal range. After skin excision/wounding, there was no significant difference in body weight, WBC, RBC, or PLT between db/db and wild-type mice, and no significant changes were detected during the 7 days of the study (Table 1).

Plasma G-CSF concentration in db/db and wild-type mice after skin excision

Plasma G-CSF levels were determined 6 hours after skin excision (Fig. 2). Prior to the start of the study, plasma G-CSF levels were significantly lower in the db/db mice than in the wild-type mice (p < 0.01). After skin excision, G-CSF levels increased significantly in both mice; however, the levels in the db/db mice were significantly lower than those in the wild-type mice (p < 0.01).

Neutrophil infiltration and collagenase expression

Acute phase neutrophil infiltration and MMP-8 expression after skin excision were examined in serial sections.

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Representative photographs on days 3 and 5 are shown in Figure 3A. Infiltrated NIMP-R14-positive neutrophils and MMP-8-positive cells in both mice increased with time. On day 3, intracellular MMP-8 expression was detected, and it was also detected in the ECM on day 5, especially in the wild-type mice. Semi-quantitative analysis of neutrophil infiltration and MMP-8

expressing cells (Fig. 3B) showed no significant difference in the mice on days 3 and 5. However, both of them were significantly greater in wild-type mice than in db/db mice.

Colocalization of MMP-8 and NIMP-R14-positive neutrophils in wound tissue from a wild-type mouse was analyzed by double fluorescent immunostaining (Fig. 3C). Many of the NIMP-R14-positive neutrophils also stained positive for MMP-8. MMP-8-positive but NIMP-R14-negative cells were also detected. Based on their structure, these cells may be other types, such as macrophages or endothelial cells.

Collagen peptide production in wound tissue

The granulation tissue that develops after wounding is remodeled by inflammatory cell infiltration. In this process, collagen, a major ECM component, is degraded by MMPs, including MMP-8. Here, we analyzed the changes in collagen dipeptide content in wounded and normal tissues in db/db and wild-type mice (Fig. 4). The levels of Hyp-Gly and Pro-Hyp in the wounded tissue were significantly lower in the db/db mice than in the wild-type mice. Pro-Hyp levels in wounded tissue gradually increased in both mice during the 7-day study; however, the levels were significantly lower in the db/db mice than in the wild-type mice. In contrast, in normal tissue obtained from the same mice, Pro-Hyp levels were unchanged during the study. In addition, no changes were observed in the Hyp-Gly levels in wild-type and db/db mice during the study.

Effect of topical rhG-CSF administration treatment on wound healing

The effects of topical rhG-CSF administration (25 μg/100 μL) on wound healing were examined. After administration of rhG-CSF, no significant changes in WBC, RBC, and PLT were observed (Fig. 5A). Wound healing, as shown by the percent wound closure 5 days after wounding, was more delayed in db/db mice than in wild-type mice (Fig. 5B). However, wound closure, measured as percent wound closure, was accelerated by rhG-CSF administration in db/db mice (p < 0.05), but was decreased (although not significantly) in wild-type mice.

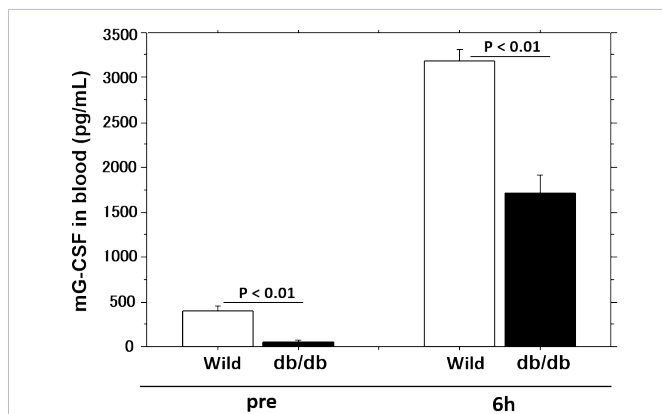The histological characteristics of the granulation tissue 7
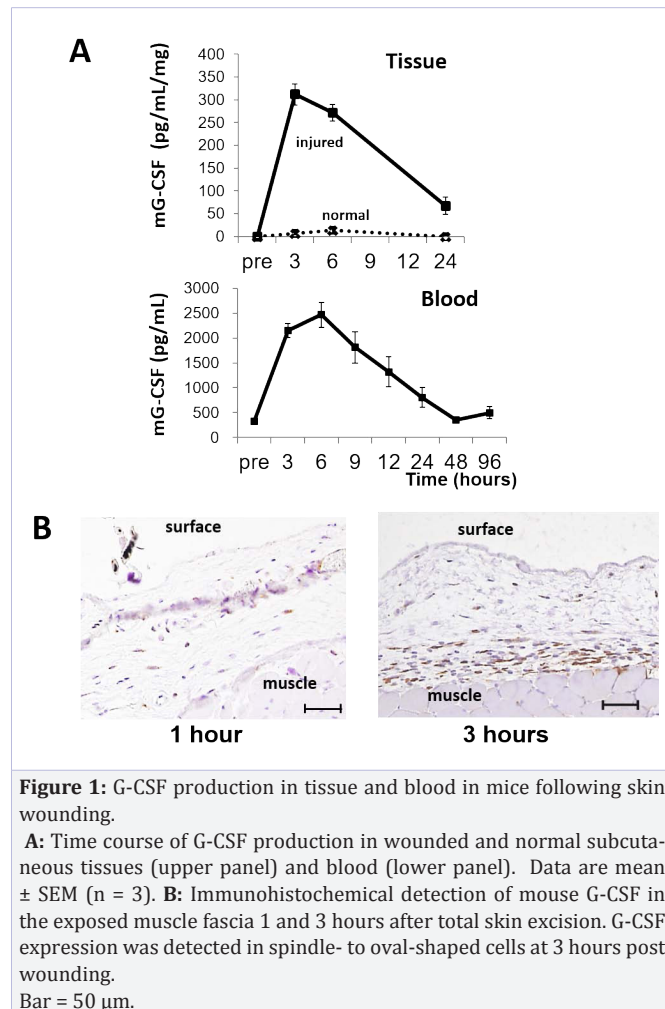
Figure 1: G-CSF production in tissue and blood in mice following skin wounding.

A: Time course of G-CSF production in wounded and normal subcutaneous tissues (upper panel) and blood (lower panel). Data are mean ± SEM (n = 3). **B:** Immunohistochemical detection of mouse G-CSF in the exposed muscle fascia 1 and 3 hours after total skin excision. G-CSF expression was detected in spindle- to oval-shaped cells at 3 hours post wounding.
Bar = 50 μm.

Table 1: Physiological data after skin excision.

	Mouse	d0	d1	d3	d5	d7
Body weight (g)	Wild	24.4±0.06	25.9±.079	24.5.±074	25.8±1.03	26.13±0.49
	db/db	48.3±0.95*	46.9±0.50*	48.1±0.90*	48.9±0.64*	49.03±0.50*
WBC (×10²/μL)	Wild	72.7±5.24	58.0±5.99	54.5±11.82	59.5±4.30	71.5±9.65
	db/db	62.01±1..02	66.3±4.91	70.7±6.12	65.5±4.44	68.0±5.00
RBC (×10⁴/μL)	Wild	979.0±8.00	893.0±46.00	894.8±31.36	887.7±12.11	907.3±26.90
	db/db	1081.7±39.84	989.3±34.07	896.7±32.10	940.0±52.60	941.3±44.21
PLT (×10⁴/μL)	Wild	45.6±7.13	59.7±14.5	67.0±7.30	59.8±6.86	65.1±4.31
	db/db	78.0±10.35	78.7±0.90	82.0±4.22	97.3±8.46	84.4±8.59

*: p<0.01 vs. wild-type, (n=3)

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Figure 2: Plasma G-CSF levels before (pre) and 6 hours after skin excision (6 h) in wild-type (wild) and db/db mice. Data are mean ± SEM (n = 3).
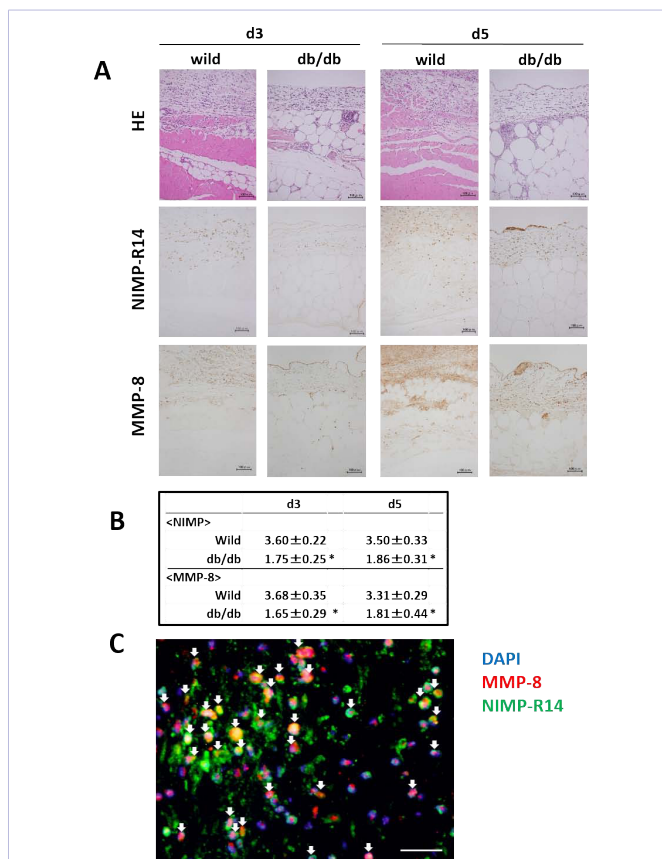
Figure 3: Neutrophil infiltration and MMP-8 expression in wild-type and db/db mice.
A: NIMP-R14-positive neutrophils and MMP-8 expression in wounded tissue after skin excision are shown by immunohistochemical staining. Bar = 100 μm.
B: Semi-quantitative analysis of NIMP-R14-positive neutrophil infiltration in wounded tissue.
*: p < 0.01. Data are mean ± SEM (n = 5).
C: Double satining of NIMP-R14 and MMP-8 in wounded tissue. Wound tissue was obtained from wild-type mice 5 days after skin excision. Tissue was doubly stained for neutrophil (NIMP-R14) and MMP-8 expression. Neutrophils with MMP-8 expression are shown by arrows. Bar = 50 μm.

days after wounding were compared (Fig. 5C). Granulation tissue in all treatment groups consisted of spindle-shaped fibroblasts, round-shaped inflammatory cells, and MT-positive collagen fibers. In wild-type mice, no apparent differences were detected between rhG-CSF-treated and untreated mice. However, in the db/db mice, thickened collagen fibers were deposited in the matrix of rhG-CSF-treated mice. Such changes were detected in 4 out of 5 db/db mice (80%).

Neutrophil infiltration and MMP-8 expression in granulation tissue after rhG-CSF administration

Neutrophil infiltration and MMP-8 expression were quantified and assessed as 1 of 6 grades. Neutrophils and MMP-8 expressing cells were scarce in unwounded subcutaneous tissue. After skin excision, the extent of neutrophil infiltration into granulation tissue was lower in db/db mice than in wild-type mice (Fig. 6A). The number of neutrophils in the granulation tissue was analyzed 3 and 5 days after topical administration of rhG-SCF on the wounds. The number of neutrophils did not change or decreased slightly in the wild-type mice, but tended to increase in the db/db mice on day 5. MMP-8 expression in the granulation tissue also exhibited a similar tendency as found in neutrophil infiltration (data not shown).

Collagen peptide production after rhG-CSF administration

After topical administration of rhG-CSF on the wounds, Hyp-Gly and Pro-Hyp contents in granulation tissue was measured on day 5 (Fig. 6B). No differences in Hyp-Gly content in the db/db and wild-type mice with/without rhG-CSF administration were detected. In contrast, Pro-Hyp content in the db/db mice was significantly increased by rhG-CSF administration (p < 0.05); no such change was detected in the wild-type mice.

Discussion

It has been reported that plasma levels of G-CSF increase in response to severe inflammation in acute appendicitis [26]. It has also been reported that inflammation-related products, such as IL-1 and lipopolysaccharide, stimulate G-CSF production by fibroblasts originating from the skin [27]. In this study, we showed that G-CSF levels in wounded tissue reached a plateau as early as 3 hours after total skin excision, and spindle- to oval-shaped mesenchymal cells (probably fibroblasts) in the wounded tissue express G-CSF. The time gap between peak expression in the tissue and blood (6 hours) may be due to the time required to reach the blood stream. Thereafter, G-CSF levels in tissue and blood decreased rapidly. Such a transient but sensitive response in G-CSF production in the peripheral tissue suggests that G-CSF is an important cytokine connecting tissue injury to the organs that produce/store neutrophils.

G-CSF can strongly mobilize not only granulocytes but also hematopoietic stem cells and progenitor cells from the bone marrow, and it is clinically used for the treatment of leukocytopenia and hematopoietic stem cell collection [28]. It has also been shown that bone marrow cells have therapeutic potential for neovascularization and wound healing [29]. These
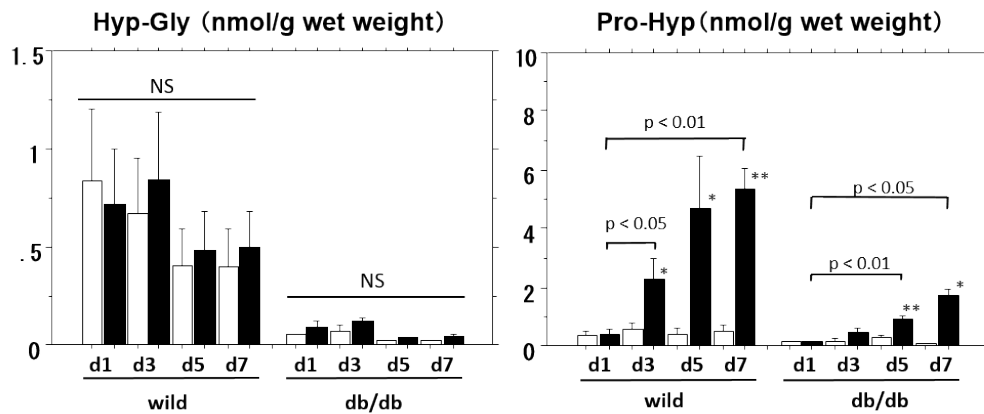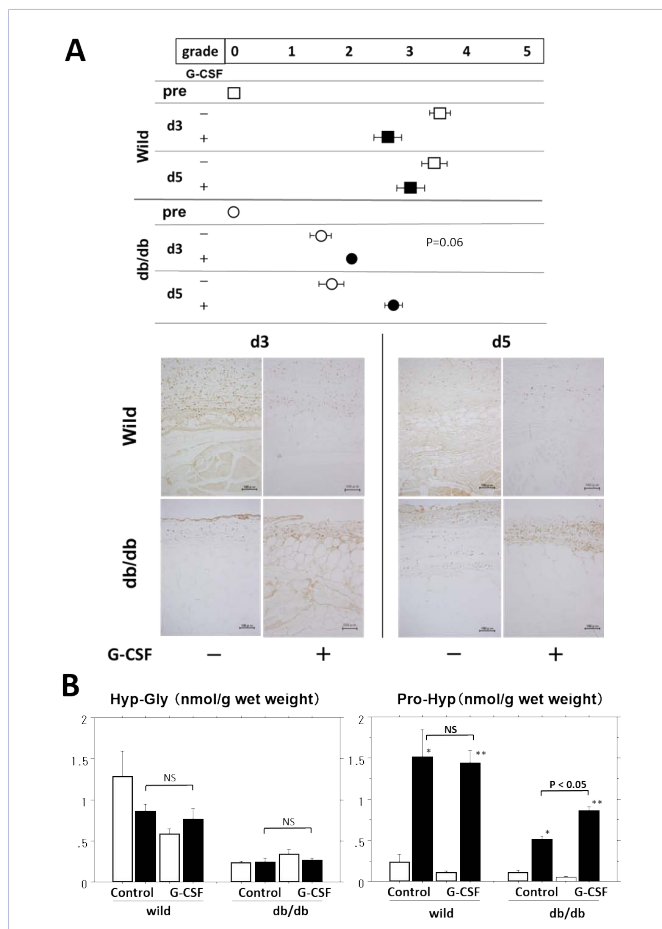
Figure 4: Changes in collagen dipeptide content in wound tissue after skin excision. After skin excision, wounded tissue (filled columns) and normal subcutaneous tissue (open columns) was obtained from the same mice. The levels of the collagen dipeptides Hyp-Gly and Pro-Hyp in wild-type and db/db mice was measured on days 1, 3, 5, and 7 after skin excision. Normal tissue vs. wounded tissue; *: $p < 0.05$, **: $p < 0.01$. Data are mean ± SEM (n = 3).

A Hematological data of wounded mice with and without topical rhG-CSF administration.

		G−CSF	d0	d3	d5
WBC (× 10²/μL)	Wild	−	84.5±7.82	70.1±4.45	54.3±14.33
		+	75.3±8.28	79.1±8.06	56.7±13.13
	db/db	−	69.8±6.14	89.8±7.95	79.2±5.06
		+	78.2±10.05	94.0±4.95	67.1±5.37
RBC (× 10⁴/μL)	Wild	−	955.7±23.91	815.9±86.27	775.3±51.84
		+	970.3±24.56	914.4±30.48	824.0±5.13
	db/db	−	980.1±38.18	911.5±11.92	912.8±22.54
		+	1020.3±61.27	945.7±31.52	936.5±28.92
PLT (× 10⁴/μL)	Wild	−	75.3±5.55	76.2±13.99	68.8±13.62
		+	68.9±9.99	69.0±12.19	71.1±9.55
	db/db	−	97.6±6.12	80.7±10.84	88.0±7.80
		+	115.0±3.25	92.5±10.96	70.0±9.77

B

C
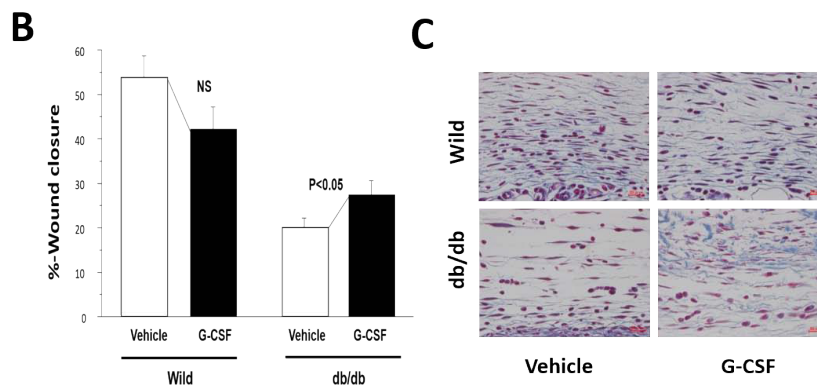
Figure 5: The favorable effect of rhG-CSF administration on wound healing in db/db mice. After skin excision, rhG-CSF (filled columns) or vehicle alone (open column) was topically administered on the wound of wild-type and db/db mice. A: Hematological data, B: percent wound closure 7 days after wounding, and C: developed granulation tissue stained by MT staining. Data are mean ± SEM (n = 5). Bar = 20 μm.

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Figure 6: The effect of rhG-CSF administration on neutrophil infiltration and collagen peptide production. After skin excision, rhG-CSF (filled) or vehicle alone (open) was administered once, and db/db (circles) and wild-type (squares) mice were sacrificed on days 3 and 5.
A: The number of neutrophils infiltrated in the granulation tissue on days 3 and 5 was graded (see Materials and Methods). Data are mean ± SEM (n = 5). Representative photographs are also shown. Bar =100 μm.
B: After skin excision, wounded (filled columns) and normal subcutaneous (open columns) tissue samples were obtained from the same mice. Hyp-Gly and Pro-Hyp content was measured on day 5. Normal tissue vs. wounded tissue; *: p < 0.05, **: p < 0.01. Data are mean ± SEM (n = 3).

number of WBCs between these mice, and the numbers did not change after skin excision. However, the number of infiltrated neutrophils in the granulation tissue of db/db mice was lower than that in the tissue of wild-type mice. These results suggest that infiltration capacity may be impaired in the diabetic mice. Moreover, in contrast to the wound healing in wild-type mice, wound healing in db/db mice was accelerated by topical rhG-CSF administration, which was accompanied by an increase in neutrophil infiltration. These findings indicate that neutrophil infiltration after wounding is an essential initial event for subsequent healing. However, why neutrophil infiltration was decreased or unchanged after rhG-CSF administration in the wild-type mice is unclear, although a negative feedback mechanism may be involved, since nondiabetic mice secrete enough G-CSF to induce neutrophil infiltration.

The roles of neutrophils in wounded tissue are not only killing bacteria and phagocytizing unfavorable materials but also degradation of the ECM. The ECM is simultaneously remodeled through the corporative action of neutrophils and fibroblasts [18]. It has been reported that the degradation products of orally ingested collagen appear in the blood as dipeptides, such as Pro-Hyp and Hyp-Gly [22], and oral intake induced a physiologically favorable effect on the skin [23]. In our study, Pro-Hyp levels in granulation tissue gradually increased during the 7 days after wounding, whereas Hyp-Gly levels did not. Different mechanisms may exist in the digestive tract and wound tissue in terms of the inherent metabolism in different organs. The specific production of Pro-Hyp in wounded tissue is first addressed in this study. We hypothesized that Pro-Hyp may be produced by collagen digestive enzymes in wounded tissue. Therefore, we examined MMPs expression. In analysis using serial sections, its expression pattern was quite similar to that of NIMP-R14-positive neutrophils in wound healing process with/without G-CSF administration. Infiltrated neutrophils primarily expressed MMP-8, but other types of cells, such as endothelial cells, also expressed MMPs. Pro-Hyp production was lower in the db/db mice, which was accompanied by a decrease in neutrophil infiltration and MMP-8 expression. Furthermore, Pro-Hyp production in the db/db mice was significantly increased by rhG-CSF administration, which was also accompanied by an increase in neutrophil infiltration and MMP-8 expression. These results suggest the involvement of Pro-Hyp in the wound healing process.

The granulation tissue that developed in normal and db/db mice after skin excision was quite different; normal granulation tissue was observed in wild-type mice, which has an organized fibrous structure. However, in db/db mice, many lipid-filled adipocytes with cholesterol clefts in the small amount of ECM were observed. Under diabetic conditions, the defect in inflammatory cell infiltration in the wounded tissue may be due to a decrease in not only their attachment to endothelial cells and migratory capacity [28] but also ECM quality, including glycation of collagens, which may acquire resistance to MMPs [32].

It has been shown that the growth of cultured dermal fibroblasts is stimulated by Pro-Hyp [24, 33], and that keratin gene expression in keratinocytes cocultured with fibroblasts

previous findings suggested the possible utility of additional G-CSF supplementation. Therefore, many interventional trials and double-blinded trials using G-CSF had been performed, especially for acute myocardial infarction and diabetic foot ulcers [15, 16]. However, its effects are still controversial. It is known that capacity of neutrophils from diabetic wounds to produce reactive oxygen species is deteriorated [30], and that in diabetic patients with foot infections, reactive oxygen species production was restored by G-CSF administration [31]. In addition, delayed wound healing in neutropenic patients was also improved by administration of G-CSF [14]. This evidence suggests that G-CSF has beneficial effects on wound healing, which may involve augmentation of neutrophil numbers and/or function.

Plasma levels of G-CSF were significantly lower in the db/db mice than in wild-type mice. There was no difference in the initial

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is also increased by the addition of Pro-Hyp [34]. In addition, osteoblastic differentiation of MC3T3-E1, accompanied by an increase in *Runx2* and *Col1a1* gene expression, is induced by Pro-Hyp [35]. These *in vitro* studies may also support our *in vivo* findings.

Conclusion

The present study shows that delayed wound healing in diabetic mice may be related to the decreases in G-CSF secretion, inflammatory cell infiltration, and Pro-Hyp production after wounding. Supplementation of diabetic wounds with G-CSF accelerated wound healing and Pro-Hyp production. Therefore, Pro-Hyp produced in wound tissue may play a central role in healing. The more precise mechanisms underlying G-CSF-induced wound healing should be clarified. Clarification of the role of Pro-Hyp in diabetic wound healing is also an important challenge, and one which we are currently pursuing for our next study.

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Declarations

All of the authors disclose NA for Conflict of Interest on this work.

Ethical Approval: All animals used in this study received humane care. This animal study was approved by the Fukuoka University Animal Experiment Committee (No. 1212618). Study protocols were in compliance with the institution's animal care guideline.

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