Processing of CXCL12 impedes the recruitment of endothelial progenitor cells in diabetic wound healing

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Introduction

Impaired wound healing is a major complication of diabetes. Although the causes are complicated and remain unclear, growing evidence suggests that high glucose levels result in elevated inflammation and poor vascular regeneration [1]. The inflammatory stage of wound repair is initiated by clotting at the site of tissue injury and subsequent release of platelets and neutrophils. The presence of neutrophils is followed by recruitment of macrophages, which are responsible for clearance of debris from the wound. Phagocytes, both neutrophils and macrophages, play a major role in the acute immune response by secreting both cytokines and matrix metalloproteinases (MMPs) [2]. However, formation of new blood vessels (angiogenesis) is indispensable for wound healing. Defects in angiogenesis, often observed in cases of diabetes, exacerbate poor blood flow and thereby impede the regenerative process in wound tissue [1,3]. In the early post-injury stage, microvascular endothelial cells are activated by hypoxia and pro-angiogenic factors including vascular endothelial growth factor [4]. Endothelial cells migrate, proliferate and establish junctions, forming new capillaries [5]. Vasculogenesis occurs in late stages after injury; this critical process, required for wound revascularization, relies on mobilization of endothelial progenitor cells (EPCs) from the bone marrow to areas of regenerating or healing tissues [6]. EPCs that are derived from bone marrow hematopoietic stem
cells may be isolated from the peripheral blood. These cells participate in both physiological and pathological neovascularization in response to certain cytokines and/or tissue ischemia, which is potentially interesting with regard to their involvement in wound healing [7]. Evidence indicates that EPC dysfunction represents a mechanism for impaired angiogenesis and subsequent poor wound healing in diabetes [8,9]. In the early and late phases of wound healing, immune cells and EPCs arrive in the wound in a sequential fashion, and have been shown to undergo significant changes to their phenotypes to play important roles in diabetic wounds. However, little is known about the interaction between these two cell types and their mechanisms of action.

EPCs are mobilized from the bone marrow into the circulation and onward to sites of ischemia. There, they undergo in situ differentiation and ultimately participate in formation of new blood vessels [10]. Cleavage of adhesive bonds by proteases such as elastase, cathepsin G and MMPs releases the EPCs from stromal cells in the bone marrow. For instance, MMP-9 has been shown to cleave the membrane-bound stem cell factor Kit ligand [11]. In injured tissue, recruitment of EPCs depends on an ischemia-induced up-regulation of CXCL12, which is released into the circulation and acts as the major chemokine responsible for recruitment of EPCs [12]. CXCL12 acts as a key regulator of angiogenesis through interaction with its receptor CXCR4 [13]. CXCL12 function has been shown to be important in diabetic wound healing because local administration of CXCL12 accelerates wound closure in a diabetic mouse model [14].

Chemokines have been reported to be processed by specific proteases, with cleavage of either their N- or C-terminal residues resulting in alterations to their structures and functions [15]. Proteolytic cleavage at the N-terminal end of a chemokine (preceding the first two conserved cysteinyl residues) specifically alters a chemokine’s actions, leading to its activation or inactivation, a switch from an agonist to an antagonist of its receptor [16], or an alteration in the chemokine–receptor binding affinity [17]. Specifically, studies have reported that CXCL12 undergoes precise proteolytic cleavage by MMP-1, 2, 3 and 9 [16]. During HIV infection, CXCL12 is converted into a highly neurotoxic protein after processing by active MMP-2. The four N-terminal residues are cleaved from the full-length protein, producing a protein that results in neuronal death and inflammation, clearly demonstrating the dramatic consequences of proteolytic cleavage on the function of CXCL12 [18]. However, it remains unclear how the function of CXCL12 is mediated in the context of inflammation in diabetes, although diabetes mellitus has been characterized by an increased levels of vascular MMPs that may result from increased oxidative stress [19].

In this study, we aimed to determine the possible mechanisms responsible for regulating the number of circulating EPCs, which may assist in diabetic wound healing. Based on the successive occurrence of inflammation and the arrival of EPCs, we hypothesize that the wound healing steps are balanced between local inflammation and vessel repair. In the streptozotocin (STZ)-induced diabetic mouse model, we found that phagocytes activated by local inflammatory cytokines in the wound inhibit mobilization and recruitment of EPCs to the lesion area. In vitro transmigration experiments indicate that phagocytes and MMPs may inactivate CXCL12, thus inhibiting local chemotaxis, which is critical to the homing of EPCs. Administration of MMP inhibitors, both in vitro and in vivo, may increase the number of EPCs present in the wound region, where inflammatory cytokines delay or exacerbate lesion recovery. Taken together, our data suggest that localized inhibition of MMPs may protect CXCL12 from cleavage and inactivation, thereby restoring the defective recruitment of EPCs to wounds. MMP inhibitors may therefore potentially be used as a novel local treatment for the poor wound healing characteristic of diabetic patients.

Results

Delay and reduction in circulating EPCs in the wound healing process of diabetic mice

In diabetic patients, the numbers of circulating EPCs are decreased, and the inflammatory stage, during which macrophages are present, is prolonged [20]. We examined the correlation between the presence of phagocytes and the mobilization of EPCs in the STZ-induced diabetic mouse model. To test our hypothesis, we created skin wounds in diabetic LysM eGFP mice and assessed the dynamics of wound healing. The percentage of initial wound size was measured daily by digital photography for 7 days after wounding, and the results were analyzed using IMAGEJ software.

From the healing curve (Fig. 1A), we observe that wound healing was dramatically impaired in the diabetic mice. Interestingly, these mice showed a sustained wound size, especially in the initial stages. Obvious healing occurred from day 4 onward. However, the
wound healing process was much longer in the diabetic mice, and did not reach the levels observed in the non-diabetic control mice (Fig. 1A). Of the monocytes isolated from the blood of the wounded mice, the circulating Sca-1\(^+\)/CD45\(^+\)/VEGFRb\(^+\) EPCs were analyzed by flow cytometry. In non-diabetic mice, we clearly observed EPCs at day 2, with increased numbers in the late stages. In contrast, the diabetic mice showed a dramatic reduction of circulating EPCs in both early and late stages, indicating impairment of circulating EPCs in the wound healing process of these mice (Fig. 1B).

EPC recruitment depends on local tissue ischemia induced by up-regulation of CXCL12. CXCL12, when released into the circulation, acts as the major chemokine for recruitment of EPCs. Correlated with the reduction of circulating EPCs, we observed reduced serum levels of CXCL12 in the diabetic mice, most significantly at day 5 (Fig. 1C), suggesting that the chemotaxis and recruitment of EPCs are defective in diabetic wound healing.

Activity of phagocytes and EPCs in the vasculogenesis of wound healing

We observed more eGFP\(^+\) phagocytes at the onset of wound healing and in the late phases of wound healing in the wound tissue of diabetic mice. These cells disappeared by day 5 in the non-diabetic wounds but their presence was sustained in the diabetic wounds. The observation that eGFP\(^+\) phagocytes appear immediately after injury but disappear within 4–5 days correlated with an increase in the number of EPCs in the circulation (Fig. 2), suggesting a chronological order between the appearance of phagocytes and EPCs during the wound healing process. These data, together with the prolonged healing process and the reduced numbers of circulating EPCs and CXCL12 chemokines in the diabetic mice, raise the possibility that the prolonged presence of phagocytes may perturb the chemotaxis of EPCs and eventually inhibit their recruitment into the circulation.
Enhanced levels of MMP-9 and pro-inflammatory cytokines

MMP-9 is an important protease released by phagocytes, resulting in acute inflammation. To study the local production of MMP-9, we dissected the wound tissue and measured the activity of MMP-9 using a gelatin-based zymography method. In agreement with the increased number of phagocytes, MMP-9 levels were significantly enhanced in tissue extracts of diabetic mice at both days 2 and 5 (Fig. 2B). Phagocytes secrete many inflammatory cytokines that interact with MMP-9 [21]. Together with the high levels of MMP-9, elevated levels of the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) were found in wound tissue extracts of diabetic mice (Fig. 3). This enhancement is in agreement with the observation of a prolonged presence of TNFα in diabetic patients, which may mediate the function of phagocytic cells [22].

Macrophages inhibit the migration of EPCs towards CXCL12 through MMP-9

To test the inhibitory role of phagocytes in recruitment of EPCs, we analyzed the in vitro transmigration of EPCs towards CXCL12. We observed that the Transwell migration efficiency of EPCs was decreased when CXCL12 was co-cultured with macrophages in the bottom of the chamber. This suggests that the inhibitory effect of macrophages on the mobility of EPCs is dependent on the proteases that process the chemokines, inhibiting the activity of CXCL12 and chemotaxis. Furthermore, this inhibitory capacity was enhanced by addition of TNFα, suggesting that macrophage activation is a critical consequence of protein cleavage (Fig. 4A). The cleavage of CXCL12 was visualized by western blotting after Tricine SDS/PAGE, which enables the full-length and cleaved forms of CXCL12 to be distinguished (Fig. 4A).

Fig. 2. Prolonged presence of phagocytes in diabetic wounds. (A) Accumulation of phagocytes at the site of the wound. Wound tissue accumulating with phagocytes were harvested at days 2 and 5, and analyzed by fluorescent microscope. Recruited phagocytes were identified as LysM eGFP+ cells. (B) Proteins were extracted from wound tissue. MMP levels were measured using gelatin zymography, and the bands were quantified using IMAGEJ software.

Fig. 3. Cytokine levels are correlated with the level of local inflammation. ELISAs demonstrated that the levels of the cytokines TNFα and IL-6 are enhanced in diabetic wounds. Each group represents five mice. Proteins were extracted from wound tissue. Asterisk indicate statistically significant differences (*P < 0.05; **P < 0.01; ***P < 0.001).
Because it has been shown that MMPs derived from macrophages are able to cleave and inactivate CXCL12 [16], we tested this using recombinant MMP-9 together with MMP inhibitors. We observed that use of recombinant MMP-9 inhibited CXCL12-mediated chemotaxis as well as macrophages, and this was restored by treatment with the MMP inhibitor GM6001. The ability of CXCL12 to drive chemotaxis corresponds to the cleavage of CXCL12. These data suggest that macrophages may inactivate CXCL12-mediated chemotaxis through secretion of MMPs such as MMP-9, thereby inhibiting the recruitment of EPCs (Fig. 4B).

Local administration of TNFα or MMP inhibitors aggravates or accelerates wound healing in diabetic mice

To investigate local interference in the recruitment of EPCs, TNFα was administered daily to each wound. The local TNFα slightly inhibited, but not significantly, the wound healing in the late stages but not at the onset of wound healing. This may be due to the high levels of cytokines at the onset. However, localized injection of the MMP inhibitor GM6001 resulted in a remarkable and significant enhancement of wound healing (Fig. 5), showing that phagocyte-derived MMPs are the key regulator for the recruitment of EPCs, which facilitates healing in diabetic wounds.

Discussion

In this study, we attempted to address the mechanism causing the neovascularization defects that result in poor wound healing in diabetes. The local presence of phagocytes in the early phases of wound healing suppressed vasculogenesis, which occurred through phagocyte-secreted MMPs. The MMPs cleaved and inactivated the chemokine CXCL12, thereby interfering with the chemotaxis that is responsible for recruitment and mobilization of EPCs, which resulted in delayed vasculogenesis and impeded wound healing. Additionally, through local administration of cytokines or MMP inhibitors, we were able to inhibit the homing of EPCs, thus accelerating the wound healing process.

Acute inflammation occurs directly after injury, and is critical to protection of the tissue from invading pathogens; this process involves numerous types of immune cells. Of these protective cells, phagocytes secreting MMPs play important roles in regulation of the immune response. Additionally, the release of immune response components, such as cytokines, may...
mediate the level of MMPs. When the inflammation subsides, tissue remodeling, including angiogenesis, collagen deposition, epithelialization and wound contraction, is essential for functional recovery [23]. Wound healing is therefore determined by a finely tuned balance between the acute reaction and subsequent reorganization of the locally injured tissue. In agreement with this concept, we observed the temporal disorder of localized inflammation and subsequent vessel repair in the wound healing of diabetic mice, represented by impaired mobilization of EPCs. The prolonged presence of phagocytes delays vessel repair and wound healing.

In response to the reduced oxygen tension of the wound, local endothelial cells produce a transcription factor, hypoxia-inducible factor 1, resulting in selective expression of CXCL12 in ischemic tissues; this results in increased adhesion, migration and homing of circulating progenitor cells to the site of injury [12]. During neovascularization, EPCs are mobilized from the bone marrow and into the circulation, a process that is dependent on CXCL12 signaling. CXCL12 may also be important for the mobilization of mature endothelial cells, another positive factor in the wound healing process [24]. In our study, the decreased number of EPCs in the diabetic mice was associated with lower levels of serum CXCL12, which may reflect a defect in the release of local CXCL12 into the circulation. Importantly, the function of CXCL12 is tightly regulated by localized cleavage of the protein, as previously demonstrated by an altered biological activity in response to MMP-2 cleavage during HIV infection [18]. Our current data indicate that MMP-9 is important in regulating the effects of CXCL12 in wound healing; this metalloproteinase is produced by phagocytes and induced by pro-inflammatory cytokines, indicating local modification of CXCL12 by neutrophils and macrophages. In contrast to the mobilization of EPCs, neutrophils and macrophages that originate from bone marrow hematopoietic cells migrate to sites of localized inflammation in a CXCR2- and CCR2-dependent manner [25]. The ligand of CXCR2 or CCR2 is expressed in the tissue upon injury and inflammation. In contrast to CXCL12 cleavage, MMPs have either a positive or no effect on inflammatory chemokines [25]. Moreover, the enhanced levels of cytokines in diabetic mice may result in the increased production of pro-inflammatory chemokines, accounting for the prolonged presence of phagocytes at the wound site.

MMPs act on numerous substrates and are known to mediate the destruction of tissue during tissue remodeling. It has been reported that activated MMPs cleave the bonds by which EPCs adhere to stromal cells, releasing the EPCs from the bone marrow environment and into the circulation. In MMP-deficient mice, the number of EPCs is reduced in the blood, indicating that mobilization of EPCs out of the bone marrow occurs in an MMP-dependent manner [11]. Nevertheless, local levels of MMPs have been shown to correlate with the poor wound healing observed in diabetic patients. MMP levels are gradually reduced over the course of normal wound healing, but sustained and elevated levels of MMPs are often observed in instances of impaired wound closure [26–28]. In the present study, we found that local production of MMPs by inflammatory phagocytes perturbs the mobilization of EPCs by inhibiting chemotaxis, demonstrating two contradictory effects of MMPs on the EPCs found in bone marrow and those localized to sites of tissue inflammation. These data suggest finely tuned regulation of the inflammatory process and of tissue remodeling during the wound healing process in vivo.

The locally produced MMPs are mainly derived from neutrophils and macrophages. Macrophages at the wound site represent key players that drive wound inflammation, operating as scavengers at sites of localized inflammation. Macrophages may polarize into pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. M1 macrophages secrete MMPs such as MMP-2 and MMP-9, which have the ability to inactivate CXCL12, whereas M2 macrophages promote wound healing and fibrosis through production of tissue inhibitor of metalloproteinases 1 (TIMP1) [29]. A prolonged phase of M1 macrophage polarization is often observed in diabetes [20]. Additionally, infection in chronic wounds leads to prolonged M1 macrophage activation, which results in perturbation of CXCL12 levels and the subsequent impairment of EPC recruitment, eventually resulting in delayed wound healing. Furthermore, both in vivo and in vitro data show that high glucose levels stimulate macrophages to enhance the production of pro-inflammatory cytokines such as IL-6 and TNF-α [30]. Additionally, it is known that TNF-α stimulation of macrophages increases the production of MMPs [30], which is in agreement with our data showing that the pro-inflammatory cytokines may trigger the macrophage response and therefore enhance the damage mediated by CXCL12 function.

Taken together, the results of our study provide insight into the link between local inflammation and vasculogenesis, revealing that dysfunction of CXCL12 regulation may occur in diabetic wounds. The secretion of phagocyte-derived MMPs is mediated by localized inflammation and is responsible for the regulation of CXCL12 function. Moreover, our findings provide a pre-clinical rationale for further studies. Localized
administration of MMP inhibitors may restore functional wound healing and may be helpful in development of therapeutic drugs for poor wound healing in diabetic patients.

**Experimental procedures**

**Mice**

All procedures were performed with approval from the Chinese PLA General Hospital. Six- to 12-week-old wild-type (C57BL/6) LysM eGFP mice were obtained from the Jackson laboratory [31]. Mice were anesthetized using an intraperitoneal injection of 80 mg·kg$^{-1}$ ketamine (Sigma, St. Louis, MO, USA) and 20 mg·kg$^{-1}$ xylazine (Sigma). For local wound injections, recombinant TNFα (10 ng·wound$^{-1}$) or the MMP inhibitor GM6001 (10 µg·wound$^{-1}$) (R&D systems, Wiesbaden, Germany) was reconstituted in NaCl/Pi and injected into the base of the wound.

**STZ-induced diabetic mouse model**

Diabetes was induced in 6–12-week-old mice by daily treatment with 60 mg·kg$^{-1}$ STZ (Sigma) in 50 mm sodium citrate (pH 4.5) for 7 days. Citrate buffer was injected in the control group. A glucometer was used to test serum glucose from the mouse tail vein. Prior to the following experimental application, including flow cytometry analysis etc, the serum glucose of all mice was measured every day for 1 week after the value reached 300 mg·dl$^{-1}$.

**Flow cytometry analysis of circulating EPCs**

Mice were anesthetized as described above. Mononuclear cells were separated from peripheral blood (0.5–1 mL) by centrifugation (10 min at 800 g at room temperature) on a Histopaque 1083 (Sigma) density gradient for 30 min according to the manufacturer’s instructions. Red blood cells were lysed with ammonium chloride solution after removing the mononuclear fraction by centrifugation (15 min at 400 g at 4 °C). Before flow cytometry measurement, cells were washed three times by centrifugation at 5 min at 400 g at 4 °C in NaCl/Pi solution containing 1% albumin. The following antibodies were used for flow cytometry: Percp-cy5.5 rat anti-mouse CD45 (BD Pharmingen, San Diego, CA, USA), PE rat anti-mouse Sca-1 (BD Pharmingen) and fluorescein isothiocyanate-conjugated rat anti-mouse VEGFR2 (BD Pharmingen).

**Assessment of the wound healing model**

A 6 mm punch biopsy was used to create wounds on the ventral surface of each mouse thigh. The underlying muscle was exposed after removing full-thickness skin. A digital camera (Canon, Tokyo, Japan) with a microscope adaptor was used to record the initial wound surface areas and the healing of wounds. The percentage of the original wound size was evaluated using ImageJ software (http://rsb.info.nih.gov/ij/index.html) for 7 days, after which time the mice were killed.

**ELISA**

A Quantikine mouse CXCL12 ELISA kit (R&D systems) was used to test the concentration of CXCL12 in the mouse serum according to the manufacturer’s instructions.

**Immunofluorescence microscopy**

Wound tissues were isolated and fixed in 1% paraformaldehyde for 1 h. Specimens were examined using an LSM700 microscope (Zeiss, Jena, Germany) and documented using a camera (Hamamatsu, Hamamatsu, Japan). Images were analyzed using ImageJ software.

**Gel zymography and cytometric bead array**

Samples of skin tissue measuring 1 x 1 cm were collected from the wounds. Protein was extracted from these samples by homogenization in RIPA buffer (25 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1% NP-40 (Nonidet P-40, octylphenoxypolyethoxyethanol), 1% sodium deoxycholate, 0.1% SDS), and subsequently separated under non-reducing conditions on 10% polyacrylamide gels containing 1 mg·ml$^{-1}$ gelatin. Equivalent amounts of total protein were used from tissue extracts. Gels were washed in Tris-buffered saline containing 2.5% Triton X-100, followed by Tris-buffered saline containing 5 mM CaCl$_2$ and 0.02% Brij 35 (Tricosaethyleneglycol mono-n-dodecyl ether), and incubated overnight in the same buffer. Gels were stained with Coomassie brilliant blue and destained in acetic acid/methanol/dH$_2$O (10 : 50 : 40).

TNFα and IL-6 levels from tissue extracts were measured using FlowCytomix Simplex kits (eBioscience, San Diego, CA, USA).

**Culture of EPCs and macrophages**

A 25 gauge needle was used to flush out bone marrow from the tibia and femurs by vigorous pipetting. The bone marrow was then centrifuged at 5 min at 400 g at 4 °C. Red blood cells were prepared as described above. Cells were plated in plates coated with vitronectin (Sigma-Aldrich, St. Louis, MO, USA) at 8.5 × 10$^5$ cells cm$^{-2}$ in EGM-2 medium (Lonza, Walkersville, MD, USA) containing human fibroblast growth factor, insulin-like growth factor, ascorbic acid, human epidermal growth factor,
heparin, vascular endothelial growth factor, hydrocortisone and 5% fetal bovine serum at 37 °C and 5% CO2. Non-adherent cells were removed, and EGM-2 was exchanged daily for fresh medium after 4 days. All molecular assays used cells harvested after day 7. Mice were injected intraperitoneally with 4% Brewer thioglycollate (Sigma-Aldrich), subjected to peritoneal lavage, and macrophages were purified by plating on Petri dishes.

Transmigration of EPCs

Migration was examined using a modified Transwell assay. A Transwell chamber (Costar, Corning, NY, USA) with a 6.5 mm diameter polycarbonate filter with an 8 μm pore size was used for this assay. The outside of the filter was coated with 10 μg/ml fibronectin overnight. Recombinant mouse CXCL12 (Promokine, Heidelberg, Germany) was then added to the lower chamber, pre-incubated with 1 x 10^6 isolated macrophages, with or without TNFα (50 ng/ml). In some cases, the lower chamber was pre-incubated with activated MMP-9 (50 ng/ml, R&D systems) or GM6001 (50 μM, Millipore, Billerica, MA, USA) for 2 h. Bone marrow-derived EPCs (1 x 10^4) were placed in the upper chamber of each well. After the 12 h migration period, non-migrating cells were completely wiped from the top surface of the membrane. We measured migrating cells adhering to the underside of the filters by 0.6-diamidino-2-phenylindole staining, and quantified them using imageJ software. The results are from four independent experiments.

Western blotting by Tricine SDS/PAGE

SDS/PAGE gels containing 4–16% Tricine were prepared as described previously [32]. ProSieve Quadcolor protein markers (Lonzia) were used as a molecular size reference. Nitrocellulose membranes (0.1 μm, GE Lifesciences, Pittsburgh, PA, USA) were used for protein transfer. The CXCL12 antibody, AH794 (AbD serotec, Kidlington, UK), recognizes both the full-length and cleaved forms of the protein.

Statistics

All data are expressed as means ± SEM. Statistical analysis was performed using Student’s t test. A P value < 0.05 was considered statistically significant.

Author contributions

GF and DH contributed equally to this work. GF and DH performed all experiments and data analysis. JC designed the protocol and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

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