Aspirin delays the healing of acetic acid-induced gastric ulcer with attenuated recruitment of hematopoietic progenitor cells to ulcer granulation tissue in mice

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Objective: To examine how aspirin affects the healing of gastric ulcer and angiogenesis. Hematopoietic progenitor cells expressing vascular endothelial growth factor receptor-1 (VEGFR1) promote angiogenesis. We examined the involvement of progenitor cells during the process of ulcer healing.

Methods: Gastric ulcers were induced by the serosal application of 100% acetic acid in mice treated with aspirin (100 mg/kg, daily) or vehicle in wild-type (WT) and tyrosine kinase-deficient VEGFR1 mice (VEGFR1 TKKO).

Results: Treatment with aspirin delayed ulcer healing and inhibited angiogenesis. Aspirin suppressed the mobilization of progenitor cells expressing CXCR4+ VEGFR1+ cells in circulation and the recruitment of these cells in ulcer granulation tissue. Ulcer healing and recruitment of CXCR4+ VEGFR1+ cells were impaired in VEGFR1 TKKO as compared with WT mice. The plasma level of stromal cell-derived factor-1 and stem cell factor and bone marrow level of pro-MMP9 (pro-matrix metalloproteinase 9) were significantly reduced in VEGFR1 TKKO mice. Treatment with aspirin in VEGFR1 TKKO mice did not further interfere with the ulcer healing compared to vehicle treatment.

Conclusion: These results for aspirin were not conclusive; however, VEGFR1 signaling is required for healing of acetic acid-induced gastric ulcer with increased recruitment of progenitor cells to ulcer granulation tissue.

Key words: aspirin, gastric ulcer, angiogenesis, vascular endothelial growth factor receptor-1, mice

Abbreviations: BM, bone marrow; BSA, bovine serum albumin; COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffer solution; PGs, prostaglandins; SCF, stem cell factor; SDF, stromal cell-derived factor; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor-1; WT, wild-type

Introduction

The healing of an ulcer is a complex process, including the formation of granulation tissue, the contraction of the ulcerated tissue, angiogenesis, and re-epithelialization. All these processes are controlled by various mediators, such as prostaglandins (PGs), cytokines, and growth factors. The use of nonsteroidal anti-inflammatory drugs (NSAIDs), in particular aspirin, has been expanded to include the prophylaxis of cardiovascular and cerebrovascular diseases. However, treatment with low-dose aspirin may be toxic to the gastrointestinal tract. NSAIDs including aspirin are known to induce gastric mucosal damage and delay ulcer healing, most likely by inhibiting cyclooxygenase (COX) activity and reducing

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that VEGFR1 signaling is required for gastric ulcer healing of gastric ulcer using a domain specific knockout mouse lacking the VEGFR1 intracellular tyrosine kinase domain (VEGFR1 TKKO). The results indicated that VEGFR1 signaling is required for the healing and angiogenesis via recruitment of CXCR4+ VEGFR1+ cells to the ulcer granulation tissue.

Materials and Methods

Animals

Male C57Bl/6 mice 8 weeks of age were obtained from Crea Japan (Tokyo). VEGFR1 TKKO mice with a C57Bl6 hybrid background were developed in our laboratory.12,13 Mice were maintained at constant humidity (60 ± 5%) and temperature (20°C ± 1) on a 12-hour light/dark cycle. All animals were provided food and water ad libitum. All experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Gastric ulcers were induced by serosal application of 100% acetic acid, as previously described.8,9 The serosal area exposed to acetic acid was 23.746 mm². Animals were fed normally thereafter. Aspirin (10 mg/ml, Merck, Whitehouse Station, NJ, USA) was suspended in 5% gum arabic in physiological saline and administered orally in a suspension (0.1 ml/10 mg of body mass) twice a day.18

Stomachs were removed and opened along the greater curvature. The ulcerated area was determined by investigators blinded to the treatment arm using Image J software.

To determine the levels of VEGF-A, stromal cell-derived factor (SDF-1), and stem cell factor (SCF) in plasma and the level of pro-MMP-9 (the proenzyme form of matrix metalloproteinase-9) in BM, plasma, and BM were collected and stored at -20°C until use. Each factor was measured using a specific ELISA (enzyme linked immunosorbent assay) kit (R&D Systems, Minneapolis, MN, USA) and all measurements were performed in duplicate.

For immunohistochemistry, gastric tissue was isolated and immediately fixed with 10% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).5,18 For the evaluation of angiogenesis, immunohistochemical staining was performed using a polymer-based detection system. The specimens were incubated with anti-CD31 rabbit polyclonal antibody (1:200 dilution, Abcam) at 4°C overnight. Immunoreactive signals were detected with 3,3’-diaminobenzene. The specimens were counterstained with Mayer’s hematoxylin, dehydrated, cleared, and then mounted. Microvessel density in the granulation tissue was taken as a measure of angiogenesis, as previously described.6 The area of highest microvessel density was identified by microscopy under low power, and then the number of microvessels in a ×400 field of this area was counted.

Bone marrow (BM) cells have been shown to contain hematopoietic stem cells (HSC), and several precursor cells were reported to be useful for recovering the lost function of damaged tissues. BM derived cells could differentiate into mature hepatocytes in the livers of rodents, mice, and humans.15 The mobilization of BM induced regeneration of the stomach after ethanol-induced ulcer in a rat model.16 Furthermore, it was reported that the mobilization of CXCR4+ VEGFR1+ cells from the bone marrow to the peripheral blood, as well as the recruitment of hemangiocytes expressing CXCR4+ VEGFR1+ to the ischemic muscle induces angiogenesis and recovery from ischemic conditions.17 Based on these reports, we hypothesized that the hematopoietic progenitor cells, CXCR4+ VEGFR1+ cells, are involved in ulcer healing.

In the present study, we investigated whether or not delayed healing of acetic acid-induced gastric ulcer in aspirin-treated mice was related to the recruitment of progenitor cells expressing VEGFR1 and CXCR4. We also examined the role of VEGFR1 signaling in the healing of gastric ulcer using a domain specific knockout mouse lacking the VEGFR1 intracellular tyrosine kinase domain (VEGFR1 TKKO). The results indicated that VEGFR1 signaling is required for the healing and angiogenesis via recruitment of CXCR4+ VEGFR1+ cells to the ulcer granulation tissue.

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counted. Gastric ulcer tissue was fixed with 4% neutral buffered paraformaldehyde at 4°C for 4 hours. Cryostat sections of approximately 8 μm were cut and incubated in 1% bovine serum albumin (BSA)/phosphate buffer solution (PBS) at room temperature for 1 hour or overnight at 4°C to block non-specific binding, followed by incubation with mouse anti-CXCR-4 (1:50, BD Pharmingen, USA), -VEGFR1 (1:100, Santa Cruz Biotechnology, CA, USA), or -EGF (1:100, R&D Systems, MN, USA) primary antibody. After washing 3 times in PBS, the sections were incubated with a mixture of the following secondary antibodies for 1 hour at room temperature: Alexa Fluor® (Abcam) 488-conjugated donkey anti-rat IgG (1:500, Molecular Probes); Alexa Fluor® (Abcam) 594-conjugated donkey anti-rabbit IgG (1:500, Molecular Probes); and Texas Red-conjugated donkey anti-IgG (1:500, Santa Cruz Biotechnology). As a negative control, sections were incubated in 1% BSA-PBS without primary antibody. Images were captured using a confocal scanning laser microscope (LSM710; Carl Zeiss, Jena, Germany) or fluorescence microscope (Biozero BZ-8000 Series; Keyence, Osaka), as previously described.19,20 After double labeling, five high power optical fields (×400) were randomly selected and the number of double-positive (yellow) cells was counted in a merged channel view. At least four animals were analyzed for each marker.

Blood was drawn from the tail vein one day after the induction of gastric ulcers. The white blood cell fraction, including platelets, was obtained by Ficoll separation and analyzed by flow cytometry, as previously described.21,22 Cells were labeled with phycoerythrin-labeled anti-VEGFR1 and PerCP-labeled anti-CXCR4 isotype control antibodies (BD Pharmingen) in the presence of anti-FcR monoclonal antibody 2.4G2 (Becton Dickinson Biosciences, NJ, USA). After washing, cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson Biosciences) and small cells (with low forward scatter) were gated for peripheral blood analysis.

Statistical analyses
Data were expressed as mean ± standard deviation (SD). Student’s t-test was used for comparisons between two groups. Comparisons between multiple groups were performed using one-way analysis of variance followed by Bonferroni’s post hoc test. A P value of less than 0.05 was considered statistically significant.

Results
Gastric mucosa is known to have decreased mucosal defenses and increased susceptibility to injury by NSAIDs. Figure 1A and B show the typical appearance of gastric ulcer in vehicle- and aspirin-treated mice at day 7. The average ulcerated area was greater in the aspirin-treated mice than that in the vehicle-treated mice (9.17 ± 3.23 mm² vs. 3.37 ± 2.06 mm², respectively; P < 0.05; Figure 1C). Furthermore, treatment with aspirin significantly reduced the number of CD31-positive cells compared to that with the vehicle (Figure 1D, E). These results supported the evidence that aspirin interfered with gastric ulcer healing by suppressing angiogenesis.

It was previously reported that bone marrow-derived cells induced regeneration of the stomach after ethanol-induced ulcer in a rat model.16 CXCR4+ VEGFR1+ hematopoietic cells derived from bone marrow are also involved in angiogenesis and tissue regeneration.17,23 Therefore, we determined whether or not aspirin affects the mobilization of progenitor cells expressing CXCR4+ VEGFR1+. Flow cytometry analysis demonstrated that the percentage of mobilization of CXCR4+ VEGFR1+ cells into circulation at day 3 was reduced in aspirin-treated mice compared to vehicle-treated mice (1.37 ± 0.23% vs. 2.27 ± 0.37%, respectively; P < 0.05; Figure 2A). In addition, the local accumulation of CXCR4+ VEGFR1+ cells around the ulcer was suppressed in aspirin-treated mice compared to vehicle-treated mice (Figure 2B). These results suggested that the process of ulcer healing system might be related to the recruited CXCR4+ VEGFR1+ cells’ dependent avenue.

We subsequently examined the role of VEGFR1 signaling in the process of gastric ulcer healing. Figure 3A shows the typical appearance of gastric ulcer in wild-type (WT) and VEGFR1 TKKO mice at day 7. The size of the ulcers in VEGFR1 TKKO mice was greater than that in the WT mice. The average ulcerated area at day 7 in the WT mice was significantly reduced compared to that in the VEGFR1 TKKO mice (9.02 ± 2.82 mm² vs. 3.76 ± 1.95 mm², respectively; P < 0.05; Figure 3B). Furthermore, the number of CD31-positive cells in granulation tissues in the VEGFR1 TKKO mice was significantly suppressed compared to that in the WT mice (15.21 ± 3.61 vessels/field vs. 21.29 ± 3.51 vessels/field, respectively; P < 0.05; Figure 3C, D). These results suggested that the gastric ulcer healing and angiogenesis were dependent on VEGFR1TK signaling.

The percentage of CXCR4+ VEGFR1+ cells in the peripheral blood in VEGFR1 TKKO mice was significantly smaller than that in the WT mice (1.07 ±
Figure 1. Delay in healing of acetic-induced gastric ulcer in aspirin-treated mice at day 7

A. Typical appearance of ulcers in vehicle- and aspirin-treated mice at day 7 after the induction of acetic acid ulcer. White circle indicates ulcer lesion.

B. Hematoxylin and eosin staining results for ulcer specimens in vehicle- and aspirin-treated mice at day 7. Scale bar, 200 μm.

C. Ulcer area at day 7 in vehicle- and aspirin-treated mice. Data are means ± SD (n = 5/group). *P < 0.05 vs. vehicle.

D. Typical immunohistochemical staining for CD31 in gastric ulcer lesions in vehicle- and aspirin-treated mice at day 7. CD31 positive endothelial cells are stained brown. Scale bar, 50 μm.

E. Microvessel counts in the granulation tissues at day 7. Data are means ± SD (n = 5/group). *P < 0.05 vs. vehicle.
Aspirin delays the healing of acetic acid-induced gastric ulcer

Figure 2. Aspirin suppresses the mobilization of CXCR4+ VEGFR1+ cells to peripheral blood and ulcer granulation.

A. The percentage of CXCR4+ VEGFR1+ cells in the peripheral blood at day 3. Data are means ± SD (n = 5/group). *P < 0.05 vs. vehicle.

B. Reduced homing of CXCR4+ VEGFR1+ cells in the gastric ulcer granulation in aspirin-treated mice. Double staining of ulcer granulation tissues from vehicle- and aspirin-treated mice with antibodies against CXCR4 (green) and VEGFR1 (green). Yellow arrows indicate double-labeled cells. Nuclei are stained with DAPI (blue). All images are representative of three independent samples. Scale bar, 50 μm.
Figure 3. The effects of VEGFR1TK signaling on gastric ulcer healing and angiogenesis

A. Typical appearance of ulcers in WT mice and VEGFR1 TKKO mice at day 7 after induction of acetic acid ulcer. White circle indicates ulcer lesion.
B. Ulcer area at day 7 was suppressed in VEGFR1 TKKO mice. Data are means ± SD (n = 5/group). *P < 0.05 vs. WT mice.
C. Typical immunohistochemical staining for CD31 in gastric ulcer lesion at day 7 in WT mice and VEGFR1 TKKO mice. CD31 positive endothelial cells are stained brown. Scale bar, 50 μm.
D. Microvessel counts in the granulation tissues at day 7 in WT mice and VEGFR1 TKKO mice. Data are means ± SD (n = 5/group). *P < 0.05 vs. WT mice.
Figure 4. VEGFR1TK signaling mobilizes CXCR4+ VEGFR1+ cells into circulation and granulation tissue and increases levels of pro-angiogenic growth factors.

A. The percentage of CXCR4+ VEGFR1+ cells in the peripheral blood at day 3 in WT mice and VEGFR1 TKKO mice. Data are means ± SD (n = 5/group). *P < 0.05 vs. WT mice.

B. Reduced recruitment of CXCR4+ VEGFR1+ cells in the ulcer granulation tissue from VEGFR1 TKKO mice. Double staining of granulation sections from WT mice and VEGFR1 TKKO mice with antibodies against CXCR4 (green) and VEGFR1 (green). Yellow arrows indicate double-labeled cells. All images are representative of three independent samples. Scale bar, 50 μm.

C-F. Plasma levels of pro-angiogenic cytokines including (C) VEGF, (D) SDF-1, (E) SCF, and (F) bone marrow levels of pro-MMP-9 at 1 day. Data are means ± SD (n = 4-6/group). *P < 0.05 vs. WT mice.
VEGFR1 signaling.

0.22% vs. 1.67 ± 0.22%, respectively; P < 0.05; Figure 4A). Moreover, as shown in Figure 4B, CXCR4+ VEGFR1+ cells were located in ulcer granulation areas in the WT mice but rarely seen in the VEGFR1 TKKO. These results suggested that VEGFR1TK signaling promotes mobilization of CXCR4+ VEGFR1+ to the ulcer granulation tissues.

Increased levels of VEGF and SDF-1 have been reported to mobilize VEGFR1+ hematopoietic progenitor cells to promote tumor growth. VEGF-A levels in both WT and VEGFR1 TKKO mice were significantly increased on day 1 compared to day 0; however, there was no difference between the groups on day 1 (125.4 ± 5.88 pg/ml vs. 126.28 ± 20.63 pg/ml, respectively; P = 0.926, n = 6 per group, Figure 4C). By contrast, the plasma levels of SDF-1 in the VEGFR1 TKKO mice were significantly lower than those in the WT mice (8.24 ± 0.91 ng/ml vs. 11.42 ± 1.15 ng/ml, respectively; P < 0.05, n = 4–5 per group, Figure 4D). In addition, hematopoietic cytokines including pro-MMP-9 (Figure 4E) and SCF (Figure 4F) were measured. BM levels of pro-MMP9 and plasma levels of SCF in the VEGFR1 TKKO mice (330 ± 110 ng/ml and 209.0 ± 81.0 pg/ml, respectively) on day 1 were lower than those in the WT mice (1,035 ± 272 ng/ml and 651 ± 182 pg/ml, respectively).

Finally, to determine the importance of VEGFR1 signaling in delay in gastric ulcer healing, we treated the VEGFR1 TKKO mice with aspirin or vehicle after the induction of an acetic acid ulcer. As shown in Figure 5, there was no difference in the average ulcerated area in VEGFR1 TKKO mice treated with aspirin and those given a vehicle (9.03 ± 4.51 vs. 8.78 ± 1.93, respectively; P > 0.05). These results suggested that the aspirin-mediated delay in gastric ulcer healing is highly dependent on VEGFR1 signaling.

Discussion

The results of the present study showed that aspirin delayed gastric ulcer healing with suppression of mobilization of progenitor cells, CXCR4+ VEGFR1+ cells in the gastric ulcer area. We also demonstrated that VEGFR1 signaling plays a critical role in the inhibitory effect of aspirin on the process of gastric ulcer healing including angiogenesis and the remobilizing CXCR4+ VEGFR1+ cells in the ulcer areas. Thus, VEGFR1 signaling induces CXCR4+ VEGFR1+ cell mobilization to ulcer lesions, thereby promoting angiogenesis and gastric ulcer healing.

The gastric ulcers are divided into three stages: the active, healing, and scar stages. The active stage was associated with hypoxia, which induces expression of VEGF, VEGFR1, and VEGFR2. It was reported that the expression of VEGFR1 was higher than that of VEGFR2 during the course of gastric ulcer healing. CXCR4 known as ligand for SDF-1 also rose significantly during the healing and scar stages associated with remodeling and vascular maturation. CXCR4 is also expressed in normal human colonic epithelial cells and may play a role in the maintenance and renewal of colonic epithelium. Furthermore, in a murine model of ischemia of the hind limbs, recruited CXCR4+ VEGFR1+ cells to ischemic tissues induced blood recovery following femoral artery ligation. VEGFR1+ hematopoietic progenitors are required for the regulation of tumor metastasis and angiogenesis. We previously reported that the mobilization of CXCR4+ VEGFR1+ hematangiocytes is important for cancer metastasis formation. In the present study, we focused on the role of VEGFR1TK signaling in the recruitment of CXCR4+ VEGFR1+ cells during gastric ulcer healing.

NSAIDs reduce the levels of PGs due to inhibition of COX. We have previously reported that microsomal PG E synthase-1 (mPGES-1) enhances the ulcer healing process and angiogenesis. In addition to the inhibitory effects of aspirin on PGs, COX, and mPGES-1, we examined whether or not the recruitment of CXCR4+ VEGFR1+ cells is involved in the effect of aspirin on ulcer healing. In the present study, we have shown that reduced recruitment of CXCR4+ VEGFR1+ cells around the ulcer area was associated with delayed acetic acid-induced ulcer healing and angiogenesis in aspirin-treated mice. From these results, we clarified that CXCR4+ VEGFR1+ cells can make up predominant molecules in the enhancement of ulcer healing and angiogenesis.

Angiogenesis is indispensable for gastric ulcer healing. Angiogenic responses are switched on at the early stage of ulcer healing. We showed the expression of CD31 was significantly attenuated in VEGFR1 TKKO mice. These results supported the evidence that VEGFR1TK signaling is critical for ulcer healing by stimulating the angiogenesis response. Angiogenesis is controlled by cytokines and various growth factors such as VEGF and SDF-1. Although angiogenesis in ulcer granulation tissue was suppressed in VEGFR1 TKKO mice compared to that in WT mice, there was no difference in the plasma levels of VEGF-A between the genotypes. Because VEGFR1 TKKO mice lack VEGFR1TK signaling, this may have been caused by the deletion of negative feedback for the down regulation of VEGF. VEGF and SDF-1 induce the remobilization
progenitor cells from BM.\textsuperscript{17,20} The release of SCF through MMP-9 activation has also been reported to be a critical step in the mobilization of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells from bone marrow.\textsuperscript{17} The results of the present study showed that increased plasma levels of SCF and bone marrow levels of pro-MMP9 were suppressed in VEGFR1 TKKO, and this was associated with fewer numbers of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells in the peripheral blood and in the ulcer granulation tissue. BM-derived cells have been shown to promote regeneration of the stomach in a rat model of ethanol-induced ulcers,\textsuperscript{16} and implantation of cultured BM-derived cells accelerates the healing of gastric ulcers. These results suggest that VEGFR1 signaling mobilizes progenitor cells by regulating hematopoietic cytokines.

To clarify whether or not aspirin interferes with gastric ulcer healing is dependent on VEGFR1TK signaling, VEGFR1 TKKO mice were treated with aspirin and with a vehicle. Aspirin did not delay gastric ulcer healing in either the VEGFR1 TKKO mice treated with or not treated with aspirin (Figure 5). This suggests that the mechanism by which aspirin results in delayed ulcer healing is due to the inhibition of VEGFR1 signaling. As described above, aspirin-treated mice exhibit delayed ulcer healing and suppressed accumulation of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells in the granulation around the ulcer area. These results supported the evidence that the mobilization of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells from bone marrow is dependent on VEGFR1TK signaling, leading to the promotion of gastric ulcer healing and angiogenesis.

In conclusion, VEGFR1TK signaling is responsible for healing of acetic acid-induced gastric ulcer through recruitment of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells to the ulcer granulation. Targeting VEGFR1TK activity or local injection of CXCR4\textsuperscript{+} VEGFR1\textsuperscript{+} cells could affect ulcer healing after a diagnosis of gastric ulcer. Therefore, selective activation of VEGFR1 appears to promote gastric ulcer healing, thereby facilitating tissue repair.

References


