The role of Angiotensin II type 1A receptor signaling in gastric ulcer healing

Sakiko Yamane,1,2 Hideki Amano,1 Yoshiya Ito,1 Tomohiro Betto,1,2 Tomoyoshi Inoue,1,2 Fumisato Otaka,1,2 Wasaburo Koizumi,2 Masataka Majima1

1 Department of Pharmacology, Kitasato University School of Medicine
2 Department of Gastroenterology, Kitasato University School of Medicine

Background: Gastric ulcer is a chronic disease featured with repeated healing and recurrence. Angiogenesis is one of important factors for the healing process. Angiotensin II (ANG II) involves in cell proliferation and tissue repair. The objective of this study is to evaluate the role of ANG II type 1A on gastric ulcer healing.

Methods and Results: Gastric ulcers were induced by the serosal application of 100% acetic acid. The gastric ulcerated areas were significantly enlarged in mice treated with ANG II type 1 receptor (AT1) blocker (ARB) compared to those in vehicle-treated mice. The number of cluster of differentiation (CD31) positive cells in the ulcerated area was lower in ARB-treated mice. There was prolonged gastric healing in the AT1a knock out (KO) mice compared to that in the wild-type (WT) mice. The mRNA (messenger RNA) levels of transforming growth factor-β (TGF-β) and stromal cell derived factor-1 (SDF-1) in the ulcerated area were significantly suppressed in AT1aKO mice compared to those in WT mice. The number of accumulated CD11b+ cells, the marker for macrophages, in the ulcer granulation was lower in AT1aKO mice. Furthermore, immunofluorescence analysis revealed that CD11b+ macrophages co-stained with SDF-1 and TGF-β in the ulcerated area in WT mice had increased compared with those in AT1aKO mice.

Conclusion: These results suggested that ANG II-AT1a signaling induces gastric ulcer healing by TGF-β and SDF-1 production from accumulated macrophages.

Key words: gastric ulcer, macrophage, angiogenesis, AT1a

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; RAS, renin-angiotensin system; ANG II, angiotensin II; ACE, angiotensin converting enzyme; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β; AT1, ANG II type 1; AT2, ANG II type 2; KO, knock out; i.p., intraperitoneal; ARB, AT1 receptor blocker; H&E, hematoxylin and eosin; MVD, microvessel density; RT-PCR, reverse-transcript polymerase chain reaction; SDF-1, stromal cell derived factor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviation; WT, wild-type; mRNA, messenger RNA; CXCR, C-X-C chemokine receptor

Introduction

Gastric ulcer is a common disease induced by Helicobacter pylori infection or several other factors, including stress, smoking, and medicine taken orally of NSAIDs.1,2 The process of ulcer healing is complex and involves a number of steps that include: granulation formation, contraction of ulcer tissue, angiogenesis, and re-epithelialization.3 Those processes are controlled by a variety cytokines and growth factors. In particular, the healing process of gastric ulcers is depended on angiogenesis, the growth of new blood vessels from the existing vasculature.4 Angiogenesis plays an essential role in healing ulcers that improves...
blood supply and promotes the nutrient supply to the healing tissue. Macrophages that infiltrate into the wound area are a key regulator of the wound healing process.

The RAS is well known to be important for the regulation of the cardiovascular system. ANG II is generated by chymase or ACE. ANG II has effects on blood pressure, proliferation, inflammation, angiogenesis, and tissue repair and development. It has been reported that ANG II has a significant role as a growth factor. We have already showed that exogenous ANG II has an effect on angiogenesis. The activation of ANG II has been shown to enhance the expression of VEGF-A, SDF-1, and TGF-β. ANG II binds two major receptors: AT1 and AT2. AT1 is classified into two subtypes: AT1a and AT1b. By using AT1 receptor antagonist and AT1a receptor KO mice, we previously reported that AT1a receptor signaling is required for tumor growth and metastasis formation. Another study has shown that ACE inhibitor and AT1 antagonist suppressed neovascularization and blood flow recovery in a hind limb model. Treatment with ANG 1-7, a vasodilator with an antiremodeling effect, one of the primary products of RAS, induced gastric ulcer healing. Genetic depletion of AT1a decreased infiltration of macrophage in the stromal area in a tumor implantation model. However, the precise mechanisms regarding the contribution of AT1 receptor signaling to gastric ulcers remain to be determined. The present study was therefore designed to investigate the role of AT1a signaling in gastric ulcer healing and to explore the underlying mechanism of ulcer healing by AT1a signaling.

Material and Methods

Animals
Male C57Bl/6 N mice, 8 weeks of age, were obtained from CLEA Japan (Tokyo). AT1aKO mice, with a C57Bl/6 hybrid background, were developed in our laboratory. Mice were maintained at constant humidity (60 ± 5%) and temperature (25 ± 1°C) 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.

Induction of gastric ulcers
Mice were anesthetized by i.p. injection of mixed anesthetic agents (medetomidine 0.75 mg/kg, midazolam 4 mg/kg, and butorphanol 5 mg/kg) and awakened by i.p. injection of atipamezole (0.75 mg/kg) throughout the experiments. We previously reported gastric ulcers were induced by the serosal application of 100% acetic acid in mice. The serosal area exposed to 100% acetic acid was 33.2 mm². The animals were fed normally thereafter. ARB, candesartan (100 mg/kg/day; Takeda Chemical Industries, Osaka), was given orally every day from 7 days before ulcer induction to the sampling day.

Measurement of ulcerated areas
The stomachs were removed and opened along the greater curvature. The ulcerated area was determined by use of ImageJ software (Bethesda, MD, USA).

Immunohistochemical staining
Gastric ulcerated area tissue was immediately fixed in 10% neutral buffered paraformaldehyde. After fixation, the tissue was dehydrated with a graded series of ethanol solutions, and then embedded in paraffin. The paraffin-embedded block was sliced 3-μm sections and each section was mounted on a glass slide and either stained with H&E stain or processed for immunohistochemistry. For the latter, sections were activated using Histo VT One (Nacalai Tesque, Yokohama) and sections were preincubated with Protein Block Serum-Free (Dako, Glostrup, Denmark) and incubated overnight at 4°C with single or a mixture of two primary antibodies as: a) anti-cluster of differentiation (CD)31 antibody (1:200, rabbit polyclonal, ab28364, Abcam, Cambridge, UK), b) anti-CD11b antibody (1:1000, rabbit monoclonal, ab133357, Abcam), c) anti-TGF-β1 antibody (1:100, rat, monoclonal, MCA74G, Bio-Rad; Hercules, CA, USA), d) anti-TGF-β1 antibody (1:50, rabbit polyclonal, ab92486, Abcam), and e) anti-SDF-1 antibody (1:50, rabbit polyclonal, ab9797, Abcam).

For primary antibodies (a, b), after immersion in a 3% solution of H2O2: for 30 minutes, the sections were incubated for 30 minutes at room temperature with N-Histofine Simple Stain Mouse MAX PO* (Nichirei Bioscience, Tokyo), and were immersed in 3, 3’-diaminobenzine. The sections were counterstained with Mayer’s hematoxylin for 30 seconds and then counterstained with Mayer’s hematoxylin for 30 seconds and then dehydrated, cleared, and mounted. For primary antibodies (c and d mix, c and e mix), after washing in PBS, the sections were incubated with a mixture of the following secondary antibodies for 1 hour at room temperature: Alexa Fluor 488-conjugated donkey anti-rat IgG (1:500, Molecular Probes, Eugene, OR, USA), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:500, Molecular Probes). Images were observed and saved with an optical photomicroscope BX53/DP72 (Olympus, Tokyo) and a fluorescence microscope (Biozero BZ-9000 Series; Keyence, Osaka). For primary antibodies (a), the positive
blood vessels were counted to determine MVD. For primary antibodies (b), the positive cells in the entire field of view (×400 magnification) were counted. For primary antibodies (c and d mix, c and e mix), the double positive cells in the entire field of view (×400 magnification) were counted.

**Real-time RT-PCR analysis**

Transcripts encoding TGF-β1, SDF-1, also known as C-X-C motif chemokine ligand 12, VEGF-A, CD31, and GAPDH were quantified by real-time PCR analysis. Total RNA was extracted from gastric ulcers with TRIzol reagent (Gibco-BRL; Life Technologies, Rockville, MD, USA) and single-stranded cDNA (complementary DNA) was generated from 1 μg of total RNA by RT with ReverTra Ace (Toyobo, New York, NY, USA). Quantitative PCR was carried out with SYBR Premix Ex Taq II (Takara Bio, Shiga). The real-time PCR primers were designed using Primer 3 software (http://primer3.sourceforge.net/) based on data from GenBank. The DNA sequences of mouse primers used for real-time PCR are shown in Table 1. Data were normalized

![Figure 1](image-url)

**Figure 1.** ARB suppressed gastric ulcer healing.

A. Typical appearance of ulcers in ARB- and vehicle-treated mice on Day 7 after induction of ulcers. The red circle indicates an ulcer in granular stomach (upper panel). H&E staining of ulcer specimens from ARB- and vehicle-treated mice on Day 7 (lower panel). B. The ulcerated area on Day 7 in ARB- and vehicle-treated mice. Data are expressed as the mean ± SD (n = 7 – 8 mice); *P < 0.05 vs. vehicle.
to the level of GAPDH in each sample.

Statistical analysis
Data were expressed as mean ± SD. All statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA, USA). Student's t-test was used for comparisons between two experimental groups. P values of <0.05 were considered to be statistically significant.

Results
The effect of AT1 in gastric ulcers
First, we examined the effect of AT1 receptor on the process of gastric ulcer healing. Figure 1A,B shows the typical appearance of gastric ulcer in ARB-treated mice and vehicle-treated mice on day 7. The ulcerated area on Day 7 in vehicle-treated mice significantly reduced the size of the ulcerated area compared to that in ARB-treated mice (vehicle: 5.00 ± 0.44 mm², ARB: 7.69 ± 1.07 mm², P < 0.05, n = 7 − 8; Figure 1B).

AT1 receptor induced gastric ulcers by the enhancement of angiogenesis
Immunohistochemical analysis against CD31 revealed that MVD around the ulcerated area was lower in ARB-treated mice compared to vehicle-treated mice (vehicle: 74.6 ± 10.1, ARB: 47.4 ± 3.02, P < 0.05, n = 5; Figure 2A,B). These results indicated that Ang II-AT1 receptor induced the healing of gastric ulcer by enhancement of angiogenesis.

The effect of AT1a receptor on gastric ulcers
ANG II binds AT1 and AT2 receptor. There are two

Figure 2. ARB suppressed angiogenesis in the ulcerated areas.
A. Representative immunohistochemical staining of CD31 in a gastric ulcer lesion on Day 7. Scale bar 20 μm. B. Microvessel density in the gastric ulcerated area on Day 7. Data are expressed as the mean ± SD (n = 5 mice); *P < 0.05 vs. vehicle.
subtype receptors in AT1 receptor, AT1a and AT1b. We have previously reported that AT1a receptor induced the tumor growth and metastasis by induction of angiogenesis. Based on this, we examine the endogenous role of AT1a signaling in gastric ulcer healing by using AT1aKO and WT mice. The ulcerated area on Day 7 in WT mice was significantly reduced compared to AT1aKO mice (WT: 4.49 ± 0.20 mm², AT1aKO: 6.17 ± 0.96 mm², P < 0.05, n = 13 − 18; Figure 3A,B).

ANG II-AT1a receptor induced gastric ulcer healing by the enhancement of angiogenesis.

We then examined whether gastric ulcer healing was induced by angiogenesis or not. Immunohistochemical analysis against CD31, known as a marker for endothelial cell, showed that the density of CD31 positive vessels around gastric ulcer was significantly decreased in AT1aKO mice compared to WT mice (WT: 100.5 ± 4.54, AT1aKO: 73.8 ± 8.60, P < 0.05, n = 5; Figure 4B).

Figure 3. AT1a signaling induced gastric ulcer healing.

A. Typical appearance of ulcers in WT mice and AT1aKO mice on Day 7 after induction of ulcers. The red circle indicates an ulcer in the granular stomach (upper panel). H&E staining of ulcer specimens from WT mice and AT1aKO mice on Day 7 (lower panel). B. The ulcerated areas on Day 7 in WT mice and AT1aKO mice on Day 7 after induction of ulcers. Data represent the mean ± SD (n = 13 − 18 mice); *P < 0.05 vs. AT1aKO.
Furthermore, expression of mRNA encoding CD31 in ulcer granulation was significantly suppressed in AT1aKO mice compared to WT mice (Figure 4C). These results indicated that ATII-AT1a signaling induced the gastric ulcer healing through angiogenesis.

The effect of ANG II-AT1a signaling on enhancement of angiogenesis stimulate factor

Angiogenesis is regulated by various angiogenesis stimulate factors, such as VEGF, SDF-1, TGF-β, FGF, and so on. We evaluated whether the gastric ulcer healing enhanced angiogenesis stimulate factors or not. The expressions of mRNA encoding SDF-1 and TGF-β in gastric ulcer in AT1aKO mice were significantly suppressed compared to WT mice on Day 7 but not in VEGF (Figure 5A-C). These results suggested that the ANG II-AT1a signaling induced gastric ulcer healing by angiogenesis depended on SDF-1 and TGF-β.

The role of CD11b+ macrophages in the healing of gastric ulcers

Many reports have shown that macrophages are involved in angiogenesis. CD11b is well known as being one of the specific markers for macrophages. Based on this, we performed immunohistchemical analysis against CD11b. Immunostaining against CD11b showed that CD11b+ cells accumulated around the ulcerated area (Figure 6A) and the number of those cells was lower in AT1aKO mice compared to those in WT mice (WT 201.8 ± 19.3 cells/field, AT1aKO 135.5 ± 7.14 cells/field, P

![Figure 4. Suppressed angiogenesis in the ulcerated areas in AT1aKO mice](image)

A. Representative immunohistochemical staining of CD31 in gastric ulcer lesions from WT mice and AT1aKO mice on Day 7. Scale bar = 20 μm. B. MVD in the gastric ulcerated areas in WT mice and AT1aKO mice on Day 7. Data represent the mean ± SD (n = 5 mice); *P < 0.05 vs. WT. C. Expression of CD31 mRNA in the ulcerated areas in WT mice and AT1aKO mice on Day 7. Data represent the mean ± SD (sham: n = 6−9, Day 7: n = 10−17); *P < 0.05 vs. WT.
< 0.05, n = 5−7; Figure 6B). These results indicated that ANG II-AT1a signaling induced gastric ulcer healing by the accumulation of CD11b+ macrophages.

The effect of ANG II-AT1a signaling on the expression of SDF-1 and TGF-β in accumulated macrophages

Macrophages recruited into the site of inflammation produce angiogenesis stimulate factors such as, VEGF, SDF-1, and TGF-β. We analyzed whether SDF-1 and TGF-β derived from macrophages or not. Immunofluorescence staining showed that the numbers of CD11b+TGF-β+ and CD11b+SDF1+ accumulated around the ulcerated areas were significantly lower in AT1aKO mice compared to those in WT mice (CD11b+TGF-β+ cells; WT: 131.4 ± 15.2 cells/field, AT1aKO: 82.1 ± 9.50 cells/field, P < 0.05, n = 4−5; Figure 7A,B, CD11b+SDF1+ cells; WT 191.6 ± 23.2 cells/field, AT1aKO 117.8 ± 10.5 cells/field, P < 0.05, n = 5; Figure 7C,D). These results suggested that ANG II-AT1a axis induced the gastric ulcer healing by

**Figure 5.** AT1a signaling induced expression of angiogenesis stimulated factors in the gastric ulcerated lesions.

mRNA levels of **A.** VEGF-A, **B.** TGF-β, **C.** SDF-1 in gastric ulcerated lesions from WT mice and AT1aKO mice were determined by real-time RT-PCR analysis. Data represent the mean ± SD (Sham: n = 6−9, Day 7: n = 10−17); *P < 0.05 vs. WT.
accumulation of CD11b+ macrophages that expressed SDF-1 and TGF-β.

Discussion

The objective of the present study was to clarify the role of ANG II-AT1a signaling in the healing of gastric ulcers. The results showed that endogenous AT1a signaling facilitates angiogenesis and induces gastric mucosal repair. Furthermore, AT1a signaling induced accumulation of macrophages to the gastric area, and that expressed SDF-1 and TGF-β. The data demonstrated that ANG II-AT1a signal enhanced gastric ulcer healing by the accumulation of macrophages.

RAS is essential for maintaining cardiovascular homeostasis and control sodium homeostasis and vascular resistance. Also it has been focused that ANG II is one of the regulators to induce MVD through AT1 receptors. In fact, ANG II/AT1 signaling had been demonstrated that ANG II induces neovascularization in various experimental models such as tumor, ischemic hind limb, and retina. AT1 has two subtypes, receptor AT1a and AT1b. We have shown that AT1a signaling induces tumor growth and metastasis formation by enhancement of angiogenesis. Gyires et al showed that ANG II-induced activation of central AT1 receptors mediated gastroprotective effect in rats model.25

These findings led us to focus on the role of AT1a signaling in the healing process of gastric ulcers. Gastric ulcer healing is the process of repairing lesions in the mucosa through the formation of granulation tissue. Granulation formation depends on the formation of new blood vessels, angiogenesis. Angiogenesis is the phenomenon that entails the formation of new vessels from existing vessels. The present study showed that gastric ulcer healing was impaired in ARB-treated mice. Furthermore, MVD around the ulcerated area was suppressed in ARB-treated mice. These results supported

Figure 6. AT1a signaling induced accumulation of CD11b+ macrophages in the ulcerated area.

A. Representative immunohistochemical staining of CD11b in the gastric ulcerated lesions on Day 7. Scale bar = 20 μm. B. The number of CD11b+ macrophages in the gastric ulcerated area on Day 7. Data represent the mean ± SD (n = 5–7 mice); *P < 0.05 vs. WT.
Figure 7. TGF-β+CD11b+ macrophages and SDF-1+CD11b+ macrophages infiltrated to the ulcerated lesions.

A. Double immunofluorescence staining of gastric ulcers from WT mice and AT1aKO mice was carried out using antibodies against CD11b (green), TGF-β (red), DAPI (blue) and overlay. Scale bar = 20 μm.

B. The number of TGF-β+CD11b+ macrophages in the gastric ulcerated area on Day 7. Data represent the mean ± SD (n = 4−5 mice); *P < 0.05 vs. WT.

C. Double immunofluorescence staining of gastric ulcers from WT mice and AT1aKO mice was carried out using antibodies against CD11b (green), SDF-1 (red), DAPI (blue) and overlay. Scale bar = 20 μm.

D. The number of SDF-1+CD11b+ macrophages in the gastric ulcerated area on Day 7. Data represent the mean ± SD (n = 5 mice); *P < 0.05 vs. WT.
the mechanism that the ANG II-AT1 axis induced gastric ulcer healing by the induction of microvessel formation. To examine whether endogenous AT1a signaling has an effect on gastric ulcer healing or not, we used AT1aKO mice. The present study showed that endogenous AT1a signaling induced gastric ulcer healing through angiogenesis. Angiogenesis is controlled by angiogenesis stimulating factors such as VEGF, TGF-β, and SDF-1. The results revealed that expressions of mRNA encoding TGF-β and SDF-1 were significantly suppressed in AT1aKO mice but not so with VEGF expression. These results indicated that gastric ulcer healing is stimulated by TGF-β and SDF-1 in an AT1a signaling-dependent manner. Those angiogenesis stimulating factors are derived from platelets and macrophages in injured tissues and in the mucosa of the ulcer margins.\(^{27,28}\) Macrophages play a critical role in pathogenesis of the disease by releasing cytokines and adhesion molecules.\(^{21}\)

We confirmed that the number of accumulated CD11b\(^+\) macrophages correlated with the extent of the gastric ulcer healing. SDF-1 is an important chemokine that binds to its receptors CXCR4 and CXCR7. SDF-1 is expressed in the stromal cell recruitment of monocytes/macrophages and tumorgenesis. In extramammary Paget's disease, lymphatic endothelial cells of subcapsular sinuses and lymph node-resident macrophages have the ability to produce SDF-1.\(^{29,30}\) In ischemic or damaged tissue, hypoxia-inducible factor-1 in endothelial cells results in the expression of SDF-1.\(^{31}\) Furthermore, SDF-1 is known to induce the recruitment and infiltration of CXCR4\(^+\) macrophages.\(^{32}\) Double immunofluorescence staining showed that accumulated CD11b\(^+\) macrophages were also co-stained against SDF-1. This result indicated the possibility that lymph node-resident macrophages might have infiltrated into the ulcerated areas.

TGF-β is a multifunctional cytokine that regulates cellular processes including proliferation, apoptosis, and differentiation.\(^{33}\) Previous reports showed that TGF-β accelerates healing of experimental ulcers and dermal wounds.\(^{34,35}\) TGF-β is mainly derived from macrophages, fibroblasts, and myofibroblasts in the granulation tissue of ulcers.\(^{36}\) Double immunofluorescent staining revealed that TGF-β was enhanced in the ulcerated areas and also co-stained against that of CD11b\(^+\) macrophages. These results suggested that CD11b\(^+\) macrophages that accumulated into the ulcerated areas secrete TGF-β that induced angiogenesis and the healing of ulcers. Furthermore, TGF-β can also trigger angiogenesis by inducing the expression of angiogenesis stimulating factors such as VEGF. In the present study, there was no change in the expression of VEGF between WT mice and AT1aKO mice. This result indicated that the induction of gastric ulcer healing induced by AT1a signaling depended on TGF-β and SDF-1 but not VEGF. In this study, we did not estimate what type of macrophages there were, M1 or M2, nor from where they derived. Future studies are warranted to elucidate this.

In conclusion, the present study indicated that AT1a signaling plays a critical role in healing of gastric ulcers by the accumulation of macrophages with the production of SDF-1 and TGF-β. The highly selective AT1a agonist will become a useful therapeutic tool for gastric ulcers.

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**Conflicts of Interest:** None

**References**


