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ORIGINAL ARTICLE

Clopidogrel inhibits angiogenesis of gastric ulcer healing via downregulation of vascular endothelial growth factor receptor 2

Jiing-Chyuan Luo [a,b,*], Yen-Ling Peng [a,b], Tseng-Shing Chen [a,b], Teh-Ia Huo [b,c], Ming-Chih Hou [a,d,e], Hui-Chun Huang [a,b], Han-Chieh Lin [a,b], Fa-Yauh Lee [a,b]

[a] *Department of Medicine, National Yang-Ming University, School of Medicine, Taipei, Taiwan*
[b] *Division of Gastroenterology, Taipei Veterans General Hospital, Taipei, Taiwan*
[c] *Institute of Pharmacology, National Yang-Ming University, School of Medicine, Taipei, Taiwan*
[d] *Endoscopic Center for Diagnosis and Therapy, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan*
[e] *Healthcare and Management Center, Taipei Veterans General Hospital, Taipei, Taiwan*

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vascular endothelial growth factor

Background/Purpose: Although clopidogrel does not cause gastric mucosal injury, it does not prevent peptic ulcer recurrence in high-risk patients. We explored whether clopidogrel delays gastric ulcer healing via inhibiting angiogenesis and to elucidate the possible mechanisms.
Methods: Gastric ulcers were induced in Sprague Dawley rats, and ulcer healing and angiogenesis of ulcer margin were compared between clopidogrel-treated rats and controls. The expressions of the proangiogenic growth factors and their receptors including basic fibroblast growth factor (bFGF), bFGF receptor (FGFR), vascular endothelial growth factor (VEGF), VEGFR1, VEGFR2, platelet-derived growth factor (PDGF)A, PDGFB, PDGFR A, PDGFR B, and phosphorylated form of mitogenic activated protein kinase pathways over the ulcer margin were compared via western blot and reverse transcription polymerase chain reaction. *In vitro*, human umbilical vein endothelial cells (HUVECs) were used to elucidate how clopidogrel inhibited growth factors-stimulated HUVEC proliferation.
Results: The ulcer sizes were significantly larger and the angiogenesis of ulcer margin was significantly diminished in the clopidogrel (2 and 10 mg/kg/d) treated groups. Ulcer induction markedly increased the expression of phosphorylated form of extracellular signal-regulated kinase (pERK), FGFR2, VEGF, VEGFR2, and PDGFRA when compared with those of normal mucosa. Clopidogrel treatment significantly decreased pERK, FGFR2, VEGF, VEGFR2, and PDGFRA

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

* Corresponding author. Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, 201, Shih-Pai Road, Section 2, Taipei, 11217, Taiwan.
E-mail address: jcluo@vghtpe.gov.tw (J.-C. Luo).

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expression at the ulcer margin when compared with those of the respective control group. *In vitro*, clopidogrel (10^{-6}M) inhibited VEGF-stimulated (20 ng/mL) HUVEC proliferation, at least, via downregulation of VEGFR2 and pERK.
Conclusion: Clopidogrel inhibits the angiogenesis of gastric ulcer healing at least partially by the inhibition of the VEGF—VEGFR2—ERK signal transduction pathway.

Introduction

Clopidogrel is an alternative antiplatelet agent that inhibits adenosine diphosphate (ADP)-induced platelet aggregation.[1,2] Clopidogrel does not inhibit the function of cyclooxygenases and does not induce endoscopically evident gastric mucosal injury in volunteers.[3] However, clopidogrel is not safe enough for gastroduodenal mucosa in patients with high risk for peptic ulcer bleeding.[4,5] In fact, ulcer formation is a dynamic imbalance between mucosal aggressive factors and defensive/repairing factors. When the function of defense and repairing factors is less than that of aggressive factors, mucosal injury worsens, and then finally ulcer formation develops.[6] Animal studies have shown that another platelet ADP-receptor antagonist—ticlopidine—impairs the healing of rat gastric ulcer by inhibiting the release of platelet derived growth factor (PDGF).[7,8]

The healing of gastric ulcer requires the reconstruction of epithelial structures and the underlying connective tissue, involving cell proliferation and angiogenesis.[6,9] Several growth factors have been implicated in the ulcer healing process.[10] The expression of these growth factors and their receptors are strongly increased over the ulcer margin.[10—12] In these growth factors, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), PDGF, and their receptors are mainly involved in the angiogenesis.[10,13,14]

In this study, we demonstrated that clopidogrel delayed rat gastric ulcer healing via inhibiting angiogenesis. In this regard, we compared the ulcer size, and angiogenesis of ulcer margin between clopidogrel-treated groups and controls. The expressions of proangiogenic growth factors and their receptors, and signal transduction pathways for angiogenesis over the ulcer margin were also measured and compared between clopidogrel-treated groups and controls. *In vitro*, we used human umbilical vein endothelial cells (HUVECs) to demonstrate that clopidogrel inhibited VEGF-stimulated HUVEC proliferation.

Methods

Animals and chemicals

Male Sprague Dawley rats (200—220 g) were reared in a standard laboratory environment.[9] The Committee on the Use of Live Animals in Taipei Veterans General Hospital approved the use of animals in this study (No: 97—137). The procedures followed were in accordance with institutional guidelines. Chemicals and drugs were purchased from Sigma-Aldrich (Sigma-Aldrich Biotechnology, St. Louis, MO, USA) unless otherwise specified. Clopidogrel was suspended in 1% methylcellulose vehicle for intragastric administration.

Induction of gastric ulcer

Gastric kissing ulcers were induced by luminal application of acetic acid to rats as previously described.[9] The anterior and posterior walls of the stomach were clamped together with a pair of forceps with a round ring (internal diameter 10 mm) situated between the two arms of the forceps. A 70% acetic acid solution of 0.15 mL was injected into the clamped portion via a 21-gauge needle. After 45 seconds, the acid solution was removed and the abdomen was closed.

Drug treatment and measurement of gastric ulcer

One day after ulcer induction, the rats were given intragastric clopidogrel of 2 or 10 mg/kg once daily for 5 or 10 days, respectively, to observe the effect on ulcer healing. The control rats were given 1% methylcellulose solution. The dose of clopidogrel (2 and 10 mg/kg/d) did not cause gastric mucosal injury in a previous study.[15] After treatment, the rats were sacrificed at Day 6 and 11, after ulcer induction. The size (mm^2) of ulcers on both the anterior and posterior walls was measured. In order to check the parameters of the healing process, another group of rats (intragastrically at doses of 2 or 10 mg/kg also) were sacrificed at Days 4 and 9, respectively, after ulcer induction. Gastric tissues were excised for immunohistological analysis. Gastric mucosa and submucosa over the ulcer margins were also collected and frozen in liquid nitrogen and stored at −70°C until determinations for different parameters took place. The "normal group" means the group of rats without ulcer induction and their gastric mucosa and submucosa were intact.

Determination of angiogenesis at ulcer margin and base

The microvessels at the ulcer margin and base in the granulation tissue of the submucosa were identified by immunonhistochemical staining with von Willebrand factor (vWF) antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA).[9] The microvessels stained with the antibody were quantified at the two sides of the ulcer margin and at the

base of ulcer crater in a microscopic field of 0.899 mm^2 (200×). The number of blood vessels at the ulcer margin was expressed by taking the average of both sides of the ulcer margin.[9]

Western blot for the expression of growth factors, their receptors, and transduction pathways

Gastric tissues were homogenized and centrifuged for supernatants. After concentration measuring using a protein assay kit (BCA Protein Assay Kit, Pierce, Rockford, IL, USA), proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinlidene fluoride (PVDF) transfer membranes (Millipore, Billerica, MA, USA). These membranes were then probed with antibodies against vWF, bFGF, FGF receptor 1 (FGFR1), FGFR2, VEGF, VEGFR1, VEGFR2, PDGFA, PDGFRA, PDGFB, PDGFRB, extracellular signal-regulated kinase (ERK), P38 mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and their phosphorylated forms (pERK, pP38, and pPI3K), and β-actin (Santa Cruz Biotechnology Inc) overnight at 4°C and incubated for 1 hour with secondary antibodies conjugated with horseradish peroxidase. The membrane was developed using the SuperSignal West Femto Trial Kit (Pierce) and exposed to an X-ray film. Quantitation was performed using a densitometer.[16]

Total RNA isolation and messenger RNA expression of growth factors and their receptors

The total RNA was extracted from rat gastric mucosa using the Total RNA Mini Kit (Geneaid, Taipei, Taiwan). The quality of isolated RNA was verified and quantified by NanoDrop 2000 (Thermo, Wilmington, DE, USA) measuring its absorbance at 260- and 280-nm wavelengths. Single-stranded complementary DNA (cDNA) was synthesized using the SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) and oligo(dT) primer. Reverse transcriptase- polymerase chain reaction (RT-PCR) primer sequences were designed according to the published cDNA sequence for rats from the National Center for Biotechnology Information (NCBI) GenBank and Primer 3 program.[16]

The sequences of forward and reverse primers are described in Table S1. The PCR was performed and each PCR product was electrophoresed on 2% agarose gel stained with ethidium bromide, and visualized under ultraviolet light. PCR quantization was performed using the Image-*J* system (NCBI published program).[16]

Culture of HUVECs

HUVECs were grown in M199 medium supplemented with 10% (v/v) fetal bovine serum (FBS), gentamicin sulfate (50 μg/mL), 25 U/mL heparin, and 30 μg/mL endothelial cell growth supplement (ECGS). The cells were cultivated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2). On the day before the experiment, the medium was changed to 1% FBS for starvation and cell cycle synchronization for 18 hours.

Table 1 Effect of clopidogrel on gastric ulcer healing.

Days after ulcer induction	Ulcer size (mm^2)	
	Day 6	Day 11
Control group	28 ± 5	11 ± 3
Clopidogrel 2 mg/kg/d	33 ± 4*	14 ± 3*
Clopidogrel 10 mg/kg/d	35 ± 5*	18 ± 3*

Values are mean ± standard deviation of six to eight rats per group.
*Indicates $p < 0.05$ when compared with the respective control group.

Cell proliferation assay

Cell proliferation was measured using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method.[15] After synchronization for 18 hours and treatment with different growth factors (10, 20, and 50 ng/mL bFGF, VEGF, PDGFA, and PDGFB, respectively) and/or clopidogrel (10^{-6}M) for 24 hours, cells were incubated with 2.5% MTT solution (5 mg/mL) for another 3 hours at 37°C. Thereafter, medium was aspirated, 0.04 N HCl-isopropanol was added and mixed thoroughly for 10 minutes. Color change and optical densities were determined by the MRX microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) at 570 nm.

Expression of growth factors, their receptors, and transduction pathways in HUVECs

After synchronization, the HUVEC cells were treated with different regimens and then were collected for Western blot and real-time PCR analysis. To quantify the gene expressions of VEGFR1 and VEGFR2, quantitative real-time PCR was performed using a LightCycler480 (Roche Applied Science, Indianapolis, IN, USA) and on the LightCycler 480

Table 2 Effect of clopidogrel on angiogenesis of ulcer margin and ulcer base.

	Numbers of microvessels/mm^2	
	Ulcer margin	Ulcer base
Four days after ulcer induction		
Control group	25.6 ± 4.1*	17.1 ± 3.6*
Clopidogrel 2 mg/kg/day	19.1 ± 2.9	12.7 ± 2.4
Clopidogrel 10 mg/kg/day	15.1 ± 1.8**	11.2 ± 1.1**
Nine days after ulcer induction		
Control group	17.3 ± 3.5*	12.3 ± 2.9*
Clopidogrel 2 mg/kg/d	12.6 ± 3.3	8.1 ± 2.6
Clopidogrel 10 mg/kg/d	10.5 ± 3.4**	7.8 ± 2.4**
Normal group	7.1 ± 1.1	

Values are mean ± standard deviation of six to eight rats per group. Normal group means the group of rats without ulcer induction.
*Indicates $p < 0.05$ when compared with the normal group.
**Indicates $p < 0.05$ when compared with the respective control group.

using GoTag qPCR Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions.[17] The signals from each sample were normalized to values obtained for housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was run simultaneously with the experimental samples. A comparative threshold cycle (Ct) method (△ △ Ct) was used to calculate the relative gene expressions (fold change) between test and reference samples. All the measurements were done in duplicate for each sample. The sequences of forward and reverse primers are: VEGFR1 5′-AGGCAAGCGCAGGTTCAC-3′ and 5′-AAGGCTTCGTGTCAAACTCTAGATG-3′, respectively, with a product of 130 base pairs (bp); VEGFR2 5′-CAAAGGGTG-GAGGTGACTGAGT-3′ and 5′-GTTTCCCGGTAGAAGCACTTGT-3′, respectively, with a product of 110 bp; GAPDH 5′-GGGTGTGAACCATGAGAAGT-3′, and 5′-ACTGTGGTCAT-GAGTCCTTC-3′, respectively, with a product of 135 bp.

Statistical analysis

Results were expressed as mean ± standard deviation. There were six to eight samples in each group. Differences between the means were analyzed with the Student *t* test when appropriate with Bonferroni correction to adjust for multiple comparisons in each experiment or one-way analysis of variance. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of clopidogrel on gastric ulcer healing and angiogenesis of ulcer healing

The average ulcer sizes on days 6 and 11 were larger in the clopidogrel-treated groups ($p < 0.05$, Table 1), so delayed gastric ulcer healing on days 6 and 11 after ulcer induction was noted in the clopidogrel-treated groups compared with the control group. The number of blood vessels at the ulcer margin and base was markedly increased 4 and 9 days after ulcer induction ($p < 0.05$). Administration of clopidogrel significantly decreased numbers of microvessels at ulcer base and ulcer margin in Day 4 and Day 9 after ulcer induction in a dose-related manner when compared with those of the respective control group (Table 2).

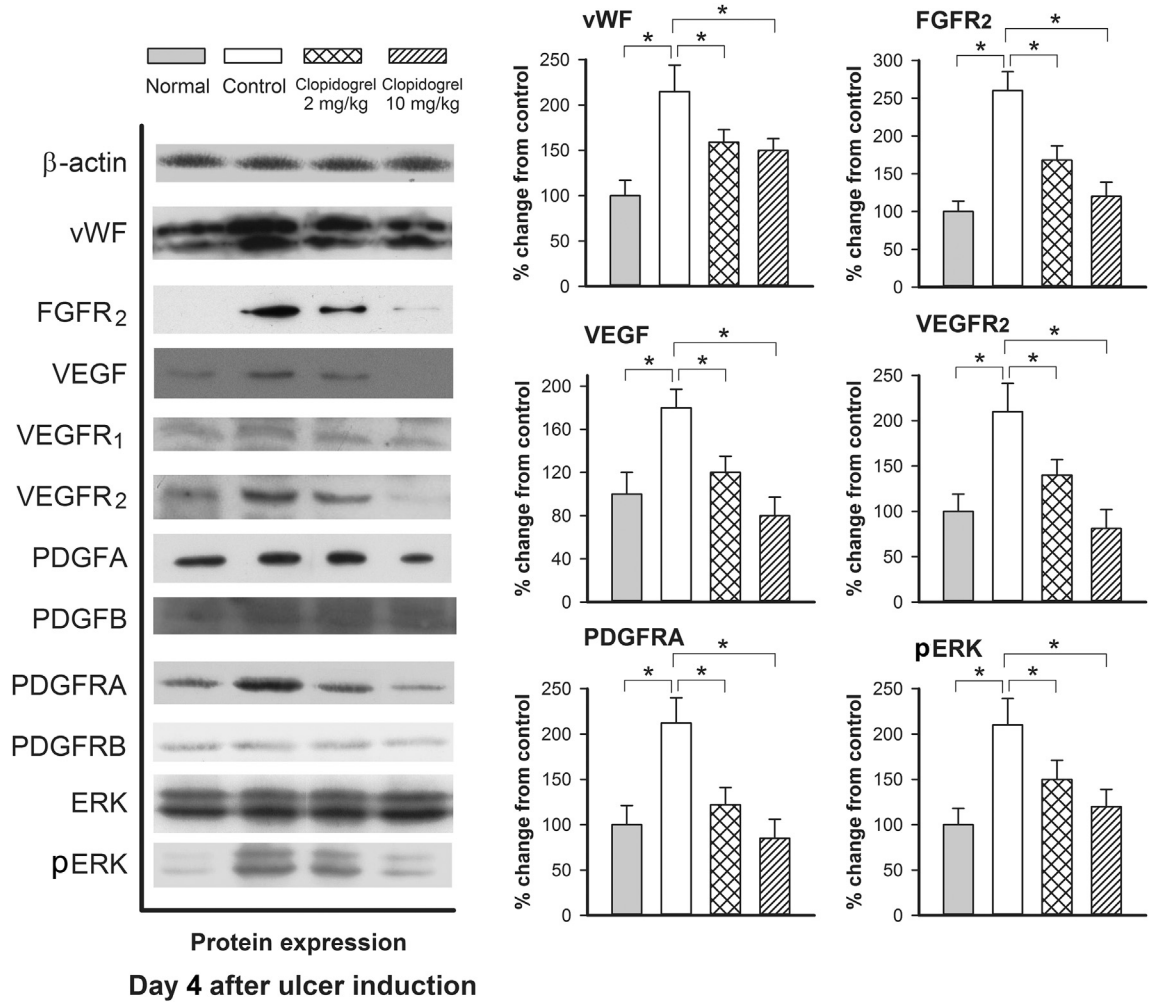

Figure 1 Effects of clopidogrel on protein expressions at Day 4 after ulcer induction. Values are mean ± standard deviation of six to eight rats per group. * Indicates $p < 0.05$ when compared with each other.

Effects of clopidogrel on protein expressions regarding angiogenesis of gastric ulcer healing

Ulcer induction markedly increased the protein expression of vWF, FGFR2, VEGF, VEGFR2, PDGFRA, and pERK at Day 4 and Day 9 after ulcer induction at the ulcer margin when compared with those of the normal mucosa. ($p < 0.05$; Figures 1 and 2). Clopidogrel treatment significantly decreased ulcer-induced expression of vWF, FGFR2, VEGF, VEGFR2, PDGFRA, and pERK at both Day 4 and Day 9 at the ulcer margin when compared with the respective control group ($p < 0.05$; Figures 1 and 2). However, the protein expression of bFGF, FGFR1, VEGFR1, PDGFA, PDGFB, PDGFRB, ERK, PI3K, pPI3K, P38, and pP38 at the gastric ulcer margin was not significantly different among the normal group, control group, and the clopidogrel-treated groups (Figures 1 and 2).

Effects of clopidogrel on mRNA expressions of growth factors and receptors of gastric ulcer healing

Ulcer induction markedly increased the mRNA expression of FGFR2, VEGF, VEGFR2, and PDGFRA at Day 4 and Day 9 after ulcer induction at the ulcer margin when compared with those of the normal mucosa ($p < 0.05$; Figure 3). Clopidogrel treatment significantly inhibited ulcer-induced mRNA expression of FGFR2, VEGF, VEGFR2, and PDGFRA at Day 4 and Day 9 after ulcer induction at the ulcer margin when compared with the respective control group ($p < 0.05$; Figure 3).

Effects of clopidogrel on growth factor-stimulated HUVEC proliferation

There was no significant difference on HUVEC proliferation (MTT method) when treated with different concentrations of bFGF (10 ng/mL, 20 ng/mL, and 50 ng/mL), or VEGF (10 ng/mL, 20 ng/mL, and 50 ng/mL), or PDGFA (10 ng/mL, 20 ng/mL, and 50 ng/mL), or PDGFB (10 ng/mL, 20 ng/mL, and 50 ng/mL for 24 hours respectively (data not shown). Samples of 20 ng/mL of bFGF, VEGF, PDGFA, PDGFB were selected for further studies. After synchronization, VEGF treatment (20 ng/mL) for 24 hours significantly increased HUVEC proliferation when compared with the controls ($p < 0.05$), whereas bFGF, PDGFA, PDGFB treatment (20 ng/mL) for 24 hours did not significantly increase HUVEC proliferation when compared with the controls (figure not shown). Although clopidogrel treatment (10^{-6}M) alone for 24 hours did not significantly decrease HUVEC proliferation

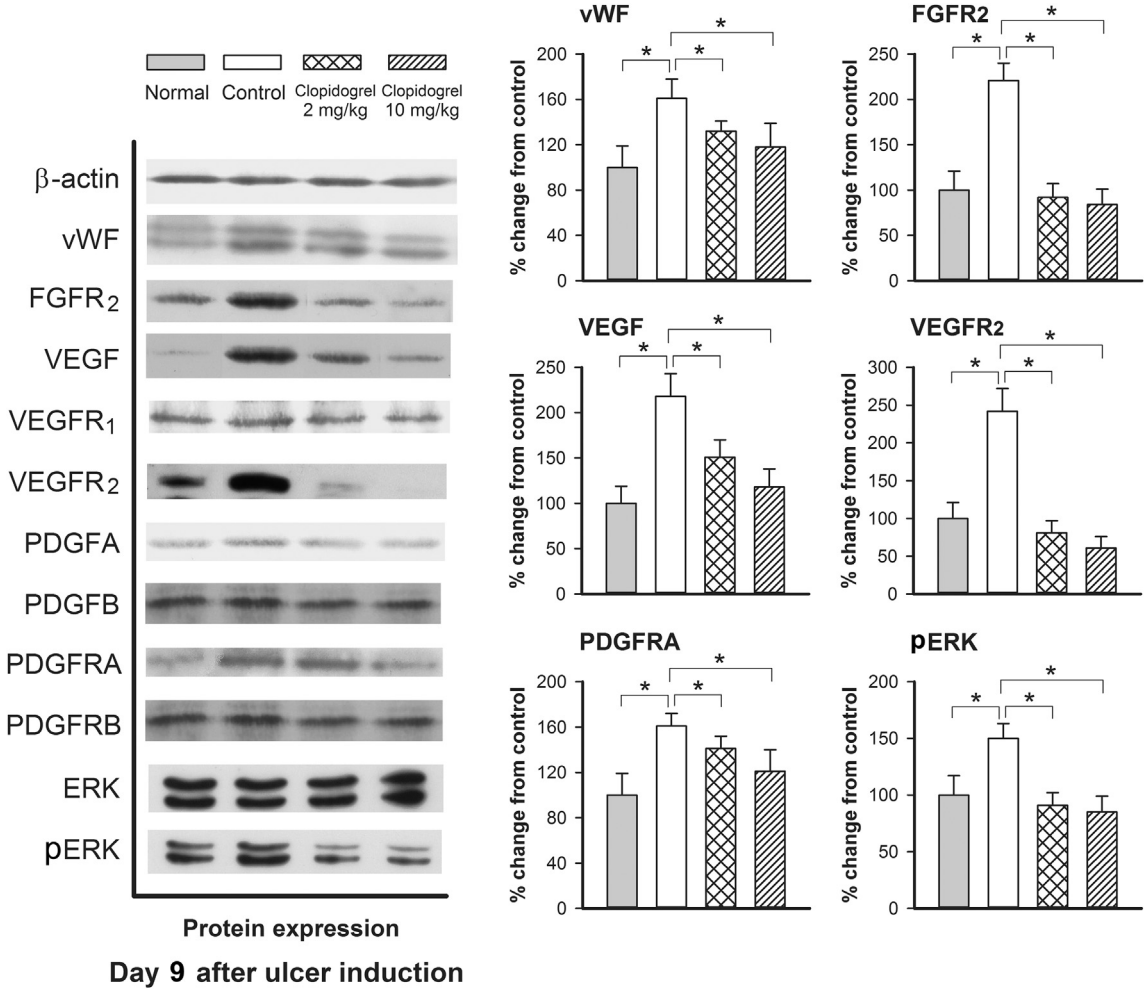

Figure 2 Effects of clopidogrel on protein expressions at Day 9 after ulcer induction. Values are mean ± standard deviation of six to eight rats per group. * Indicates $p < 0.05$ when compared with each other.

when compared with the controls, clopidogrel (10^{-6}M) significantly diminished VEGF (20 ng/mL)-stimulated HUVEC proliferation ($p < 0.05$; figure not shown).

Effects of clopidogrel on VEGF-stimulated expression of pERK and VEGF receptors

After synchronization, VEGF treatment (20 ng/mL) for 10 minutes significantly increased protein expression of pERK, VEGFR1, VEGFR2 ($p < 0.05$; Figure 4), but not ERK, PI3K, pPI3K, P38, or pP38 when compared with the controls (data not shown). However, pretreatment with 10^{-6}M clopidogrel for 3 hours, significantly inhibited VEGF-stimulated protein expression of pERK and VEGFR2 when compared with that of VEGF group. Clopidogrel alone (10^{-6}M) for 3 hours also significantly inhibited the protein expression of pERK and VEGFR2 when compared with the control ($p < 0.05$; Figure 4).

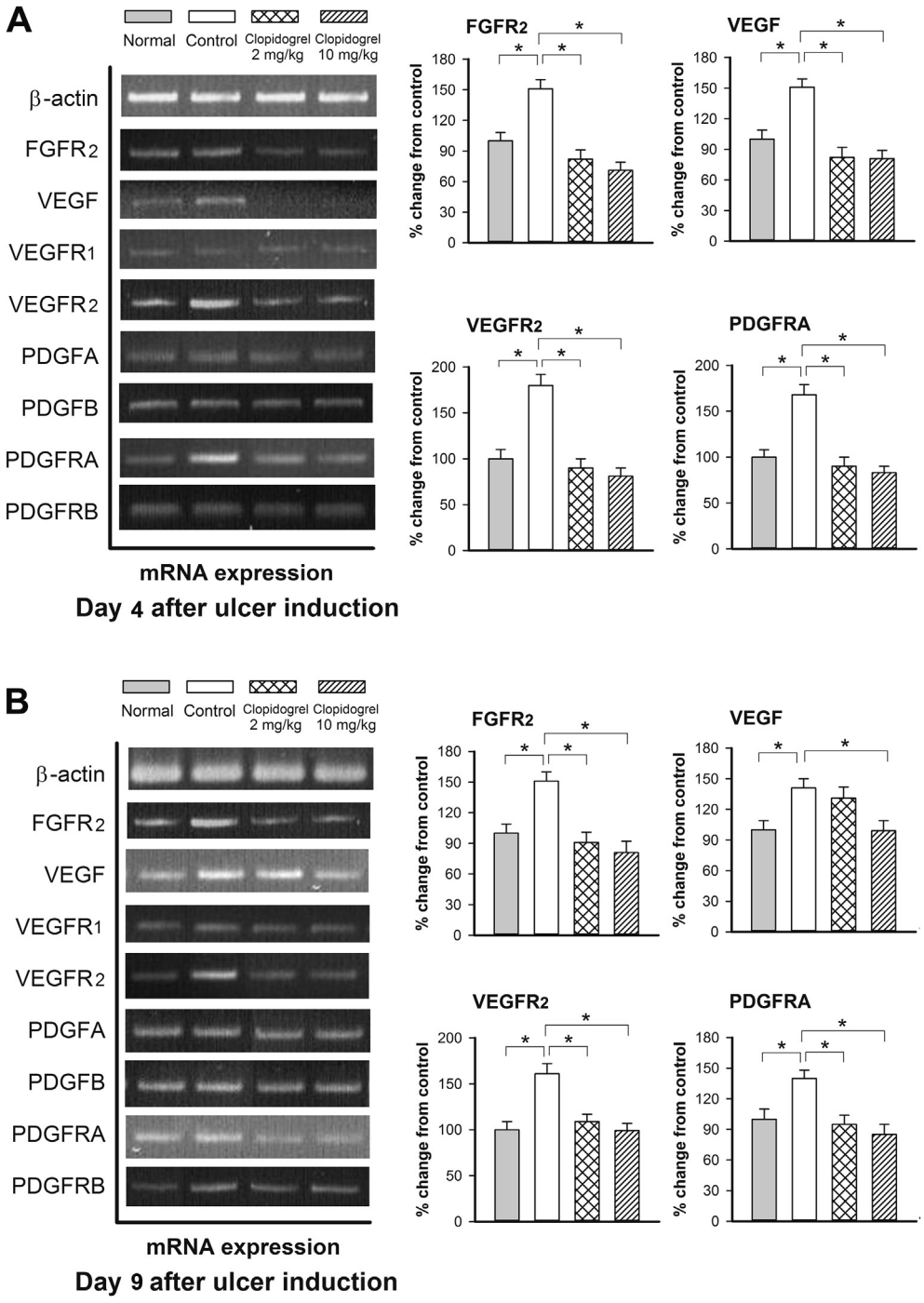

Figure 3 Effects of clopidogrel on messenger RNA expressions at (A) Day 4 and (B) Day 9 after ulcer induction. Values are mean ± standard deviation of six to eight rats per group. * Indicates $p < 0.05$ when compared with each other.

Effects of clopidogrel on VEGF-stimulated mRNA expression of VEGF receptors

After synchronization, VEGF treatment (20 ng/mL) for 10 minutes significantly increased mRNA expression of VEGFR1, VEGFR2 when compared with the controls ($p < 0.05$; Figure 5). The increase was more conspicuous on VEGFR2 expression than VEGFR1 expression. However, pretreatment with 10^{-6}M clopidogrel for 3 hours significantly inhibited VEGF-stimulated mRNA expression of VEGFR1 and VEGFR2 when compared with that of the VEGF group. The inhibition was more conspicuous on VEGFR2 expression than VEGFR1 expression. Clopidogrel alone (10^{-6}M) for 3 hours also significantly inhibited the mRNA expression of VEGFR1 and VEGFR2 when compared with the control ($p < 0.05$; Figure 5).

Discussion

In the current study, we demonstrated that clopidogrel inhibited the angiogenesis of gastric ulcer healing in rats and this was associated with diminished expression of FGFR2, VEGF, VEGFR2, PDGFRA, and pERK over the ulcer margin. *In vitro*, we showed that clopidogrel inhibited VEGF-stimulated HUVEC proliferation, at least, via downregulation of VEGFR2 and pERK. This study, for the first time, demonstrated that clopidogrel inhibited angiogenesis and delayed ulcer healing at least by the inhibition of the VEGF-VEGFR2-ERK signal transduction pathway.

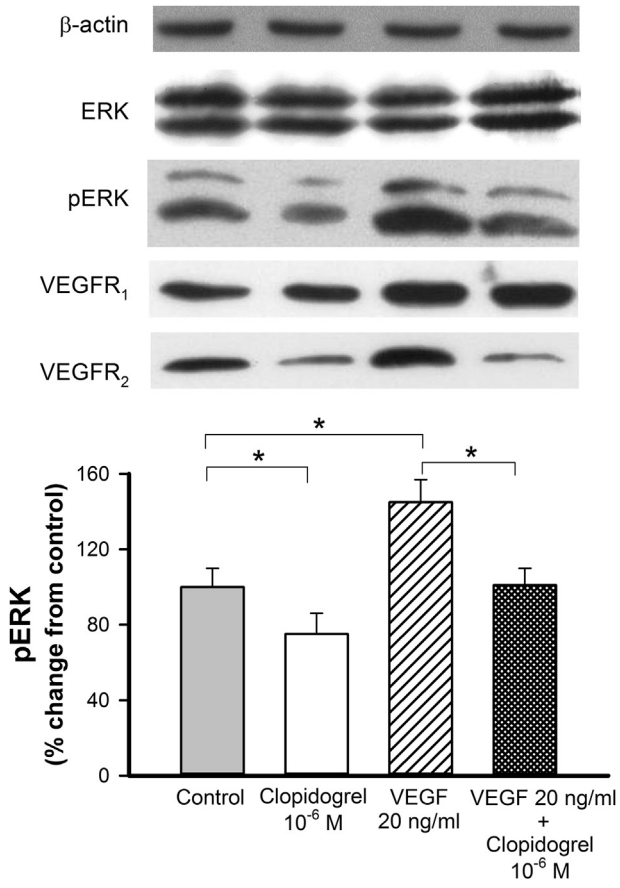

Figure 4 Effects of vascular endothelial growth factor (VEGF) with or without clopidogrel on the protein expression of VEGF receptor and phosphorylated forms extracellular signal-regulated kinase (pERK). Values are mean ± standard deviation. $n = 6$ in each group. * Indicates $p < 0.05$ when compared with each other.

VEGF is secreted as dimeric glycoproteins and recognizes specific receptors with intrinsic tyrosine kinase activity, VEGFR1 and VEGFR2.[18] VEGF is an important angiogenic mediator that acts specifically on vascular endothelial cells to increase vascular permeability, and stimulates endothelial cell proliferation, migration, and tube formation.[10,13] VEGF pathway activation significantly accelerates gastric ulcer healing by enhancing angiogenesis at the ulcer site.[19] The study showed that ulcer activated VEGF and VEGFR2 expression to promote ulcer healing physiologically, but clopidogrel treatment significantly inhibited ulcer-induced VEGF/VEGFR2-ERK expression over the ulcer margin and then inhibited the angiogenesis of ulcer healing. Our findings echo those of Ma et al,[8] which showed that ticlopidine, another ADP receptor antagonist, impaired angiogenesis and gastric ulcer healing in rats with decreasing VEGF level.[8]

In the *in vitro* part of this study, we demonstrated that VEGF activated VEGFR especially VEGFR2, then upregulated ERK/pERK signal transduction pathway, but not PI3K or P38 pathway and finally promoted HUVEC proliferation. These findings were consistent with previous studies which showed that VEGF induced HUVEC angiogenesis via stimulating the Ras-MEK-ERK pathway, which was blocked by MEK inhibitor PD98059[20,21] and that VEGF bound the VEGF receptor and activated ERK1/2 through the phospholipase γ (PLC-γ) PKC-α-B-Raf-pathway and then induced HUVEC proliferation.[22] We found that clopidogrel inhibited VEGF-stimulated HUVEC proliferation by downregulation of VEGFR2 and pERK. Angiogenesis is a multiple-step process involving cell migration, proliferation of endothelial cells and their subsequent realignment to form new capillary tubes.[23] In our *in vitro* study, only HUVEC proliferation via MTT method was assayed. Further studies to evaluate the action and mechanisms of clopidogrel on Matrigel invasion assay and migration assay are needed in the future.

This study showed that clopidogrel inhibited the angiogenesis of gastric ulcer healing and delayed rat gastric ulcer healing via inhibiting VEGF-VEGFR2-ERK signal transduction pathway. Our previous study showed that clopidogrel delays gastric ulcer healing in rats via inhibiting gastric epithelial cell proliferation, at least by inhibition of the EGF receptor-ERK signal transduction pathway. Clopidogrel delays gastric ulcer healing involving ERK signal transduction pathway and inhibits gastric mucosal cell proliferation and mucosa/submucosal angiogenesis. These findings corroborate previous clinical study that clopidogrel is not safe enough for the gastric mucosa of patients with a history of aspirin-associated ulcer or ulcer bleeding when they took clopidogrel after the ulcer had healed.[1] Furthermore, ulcer recurred at previous ulcer locations among these patients.[4,24] It seems that ADP receptor antagonists, both ticlopidine and clopidogrel, cause no gastric mucosa injury,[3,7] but they did delay gastric ulcer healing.[8,15,7]

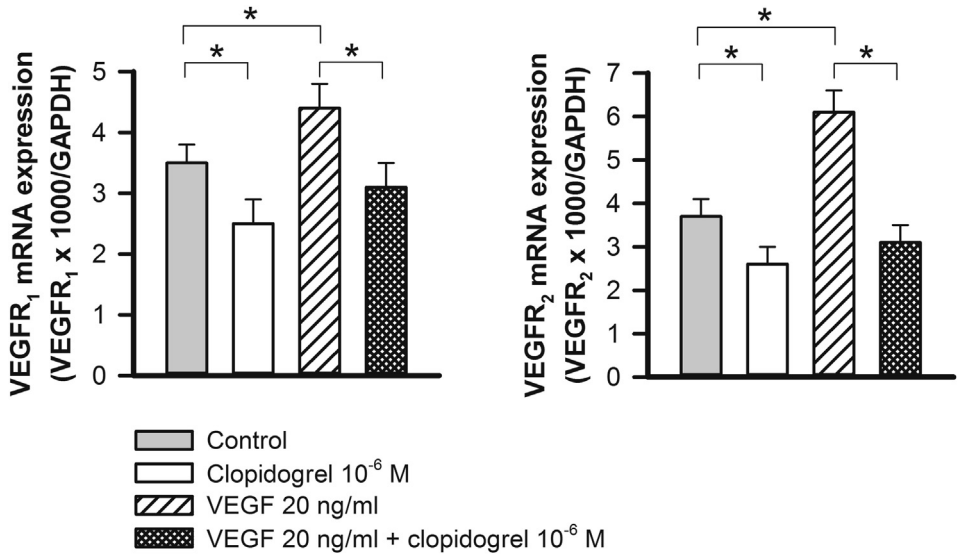

Figure 5 Effects of vascular endothelial growth factor (VEGF) with or without clopidogrel on messenger RNA expression of VEGF receptors measured by real-time polymerase chain reaction. Values are mean ± standard deviation. $n = 6$ in each group. * Indicates $p < 0.05$ when compared with each other.

In conclusion, clopidogrel inhibits the angiogenesis of gastric ulcer margin and delays gastric ulcer healing, which is associated with inhibiting VEGF-VEGFR2-ERK signal transduction pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfma.2015.07.022

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