Journal of the Formosan Medical Association (2016) 115, 764—772

Available online at www.sciencedirect.com

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journal homepage: www.jfma-online.com

ORIGINAL ARTICLE

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

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Received 19 November 2014; received in revised form 25 July 2015; accepted 27 July 2015

KEYWORDS
angiogenesis;
clopidogrel;
extracellular signal-regulated kinase (ERK);
gastric ulcer healing;
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.

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http://dx.doi.org/10.1016/j.jfma.2015.07.022

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