A vascular endothelial growth factor mimetic accelerates gastric ulcer healing in an iNOS-dependent manner

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Dudar GK, D’Andrea LD, Di Stasi R, Pedone C, Wallace JL. A vascular endothelial growth factor mimetic accelerates gastric ulcer healing in an iNOS-dependent manner. Am J Physiol Gastrointest Liver Physiol 295: 374–381, 2008. First published June 26, 2008; doi:10.1152/ajpgi.90325.2008.—Angiogenesis is crucial to all types of wound healing, including gastric ulcer healing. The most potent promoter of angiogenesis is vascular endothelial growth factor (VEGF). We hypothesized that a 15-amino acid peptide designed to mimic the angiogenic action of VEGF would accelerate gastric ulcer healing. Gastric ulcers were induced in mice by serial application of acetic acid. Treatment with the VEGF mimetic accelerated gastric ulcer healing when administered orally or intraperitoneally, at a dose of 50 ng/kg or greater. Such healing was not observed when the reverse sequence pentadecapeptide or the full-length VEGF protein was administered. Contrary to our hypothesis, the VEGF mimetic did not significantly increase angiogenesis in the ulcerated stomach. The enhancement of ulcer healing by the VEGF mimetic occurred independently of cyclooxygenase-2 (COX-2) activity but was blocked by inhibitors of inducible nitric oxide synthase (iNOS). These results demonstrate that a VEGF mimetic is a potent stimulus for gastric ulcer healing, even when given orally. The effects of the mimetic were independent of stimulatory effects on angiogenesis and COX-2 activity but were dependent on iNOS-derived NO production.

stomach; nitric oxide; cyclooxygenase; angiogenesis; prostaglandin; gastric mucosa

CHRONIC GASTRIC ULCER HEALING involves epithelial cell migration and proliferation, matrix remodeling, and angiogenesis. These processes can be modulated by a plethora of transcription factors, growth factors, and cytokines (36). Growth factors that influence angiogenesis have been of particular interest to many investigators (13, 23, 31). Vascular endothelial growth factor (VEGF), a 46-kDa homodimeric glycoprotein, is the most potent stimulator of angiogenesis (31). It is produced by a variety of cell types including macrophages, smooth muscle cells, fibroblasts, megakaryocytes, and neoplastic cells (2, 17).

Several studies have provided evidence for a role of VEGF in gastric ulcer healing. For example, expression of VEGF was detected in the margins of human gastric ulcers (32). The same group observed that rat gastric fibroblasts could express VEGF, and this expression was enhanced when the fibroblasts were exposed to growth factors that stimulated ulcer healing (e.g., epidermal growth factor and hepatocyte growth factor) (33). Expression of VEGF was observed to be elevated following induction of gastric ulcers in rats, in parallel with increases in angiogenesis (29).

There is also a link among prostaglandin synthesis by the gastric mucosa, VEGF expression, and angiogenesis. Although indomethacin suppressed ulcer healing and angiogenesis in rats, it did not affect gastric VEGF expression (29). It has been known for several years that cyclooxygenase-2 (COX-2) makes an important contribution to the healing of ulcers throughout the gastrointestinal tract (15, 20, 25, 28). Again, this may be linked to the role of VEGF in ulcer healing. COX-2 and VEGF have been colocalized in fibroblasts in the ulcer bed (19). A selective COX-2 inhibitor suppressed VEGF release from human gastric fibroblasts, and this could be reversed by addition of prostaglandin E2 (PGE2) to the culture medium (19).

The possibility that VEGF can be exploited therapeutically to improve gastric ulcer healing has been investigated. Jones et al. (10) observed enhanced ulcer healing in rats following a single injection of naked DNA encoding VEGF. This effect was significantly reduced by cotreatment with an antibody directed against VEGF. We exploited the platelet as a rich source of VEGF to examine its effects on gastric ulcer healing. Ulcer healing could be substantially accelerated by oral treatment with a suspension of human platelets, and this effect was reversed by immunoneutralization of VEGF (40).

In the present study, we have examined the effects of a 15-amino acid peptide that adopts a helical conformation in aqueous solution that resembles the α-helical region (17–25) of the VEGF protein (4). This peptide has been shown to bind to both the VEGF-1 and VEGF-2 receptors and to induce endothelial cell proliferation, activate the same signaling cascades as VEGF, and stimulate capillary formation in vitro (4). We have examined the ability of this VEGF mimetic to influence experimental ulcer healing and characterized the dependence of such effects on angiogenesis and on COX-2 and nitric oxide synthase (NOS) activity.

MATERIALS AND METHODS

Animals. All experiments were approved by the University of Calgary Animal Care Committee. Male Wistar rats weighing 150–175 g and male C57BL/6 mice weighing 20–25 g were obtained from Charles River Laboratories (Montreal, Quebec, Canada). The animals had free access to a standard laboratory chow and were housed in a room with a 12-h:12-h light-dark cycle.

Peptides. The VEGF mimetic used in these studies is a 15-amino acid peptide (KLTWQELYQLKYKGI), synthesized as described by D’Andrea et al. (4). The NH2 terminus of the peptide is acetylated, whereas the COOH terminus is capped with an amide group. The effects of a peptide with the reverse sequence of the VEGF mimetic (IGKYKLQYLEQWTLK) were also assessed. As with the VEGF mimetic, this peptide has been shown to bind to both the VEGF-1 and VEGF-2 receptors and activate similar signaling cascades as VEGF, and stimulate capillary formation in vitro (4).

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mimetic, the NH₂ terminus of the reverse peptide is capped with an acetyl group and the COOH terminus ends in an amide group.

**Gastric ulcer induction.** Gastric ulcers were induced in mice according to the method outlined by Wang et al. (45), with slight modifications (41). In brief, a laparotomy was performed under halothane anesthesia, and the stomach was externalized. A 1-ml syringe, with its barrel cut and filed smooth, was placed firmly against the serosal side of the stomach. Acetic acid [200 μl of 20% (vol/vol)] was added to the syringe such that it was in contact with the serosal surface for 1 min. The acetic acid was removed from the syringe by aspiration and replaced with several rinses of sterile saline. The stomach was then placed back to its original anatomical position, and the midline incision was closed with sutures.

The procedure used to induce gastric ulcers in rats was similar to that employed for mice, with a few differences. The rats were fasted overnight before the surgery. Acetic acid (80% vol/vol) was applied to the serosal wall of the stomach for 1 min with the barrel of a 3-ml syringe.

**Measurement of gastric ulcer area.** In each experiment, a group of mice or rats was euthanized on day 3 after ulcer induction. This provided data on the size of ulcers before treatment. Groups of animals were similarly euthanized at various times after beginning treatment with test drugs. In all cases, the stomach was excised and pinned out (mucosal surface up) onto a wax block. A 25-mm² paper grid was placed adjacent to the ulcer, and a photograph was taken. The photograph was then enlarged and used for planimetric measurement of the ulcer area (performed by an observer unaware of the treatments the animals had received). The area of ulceration was then converted into units of mm² using the paper grid as a reference. In some experiments, samples of gastric tissue were processed for further evaluation (e.g., immunohistochemistry).

**Effects of the VEGF mimetic on ulcer healing.** The ability of the VEGF mimetic to accelerate ulcer healing was first examined in the mouse. Beginning 3 days after ulcer induction, mice were treated twice-daily intraperitoneally with the VEGF mimetic (100 ng/kg) or vehicle (sterile 0.9% saline). Groups of 5–7 mice were euthanized on days 5, 7, and 10 after ulcer induction, and gastric ulcer areas were measured.

Next, the ability of different doses of the VEGF mimetic to enhance gastric ulcer healing was assessed. Beginning on day 3 after ulcer induction, mice were treated intraperitoneally with vehicle (n = 16) or the VEGF mimetic at 25 ng/kg (n = 4), 50 ng/kg (n = 13), or 100 ng/kg (n = 4). The mice were euthanized on day 7 after ulcer induction for measurement of ulcer area.

Whether or not the VEGF mimetic would be effective when administered orally was then examined. Groups of mice were treated twice daily with vehicle or the VEGF mimetic (50 ng/kg), each being given either intraperitoneally or orally (n = 5–9 per group). The mice were euthanized on day 7 for measurement of gastric ulcer area.

A series of experiments was performed to determine whether the beneficial effects of the VEGF mimetic on ulcer healing could be observed with the full-length VEGF protein. Groups of 6–9 rats were treated twice daily with VEGF (1 μg/kg) or vehicle, given either orally or intraperitoneally, beginning on day 3 after ulcer induction and continuing until day 7. The mice were then euthanized, and ulcer areas were measured. The dose of VEGF used in this experiment represents an equimolar dose to 50 ng/kg of the VEGF mimetic. The effects of the VEGF mimetic on gastric ulcer healing were then compared with the effects of a peptide with the same length as the mimetic but with the reverse sequence. Twice daily intraperitoneal treatment with vehicle (n = 6), the VEGF mimetic (50 ng/kg, n = 6), or the reverse sequence peptide (50 ng/kg, n = 7) was conducted over a 3-day period beginning on the third day after ulcer induction. The mice were then euthanized, and ulcer areas were determined.

The experiments described above involved the use of mice. The following experiment was performed to determine whether the VEGF mimetic could influence ulcer healing in rats. Beginning on day 3 after ulcer induction, groups of 5 rats each were treated twice daily intraperitoneally with the VEGF mimetic (50 ng/kg) or vehicle. The rats were euthanized on day 7 after ulcer induction for measurement of gastric ulcer area.

**Gastric acid secretion.** Since agents that suppress gastric acid secretion can accelerate ulcer healing in this model (9), we examined the possibility that the VEGF mimetic might suppress gastric acid secretion in the mouse. Mice were fasted overnight and anesthetized with halothane. The pyloric sphincter was ligated, and the mice were allowed to recover consciousness. The mice were then treated intraperitoneally with vehicle (n = 4) or the VEGF mimetic (100 ng/kg, n = 5). Three hours later, the mice were anesthetized with halothane, the lower gastro-esophageal junction was clamped, and the stomach was carefully excised. The contents of the stomach were collected into a tube and the volume of the fluid determined gravimetrically. The pH and the titratable acidity of the gastric juice were determined, as described previously (1).
mediator of VEGF-dependent angiogenesis (22, 47). Indeed, there is evidence of reciprocal regulation between NO and VEGF in the context of angiogenesis (11).

We performed studies to determine whether NO activity was required for the enhancement of ulcer healing by VEGF and further examined whether selective inhibition of the inducible isoform of NOS (iNOS) contributed to VEGF mimetic-induced ulcer healing. Beginning 3 days after the ulcer induction, groups of mice were treated twice daily intraperitoneally with saline (n = 7), the VEGF mimetic (50 ng/kg, n = 7), nitro-L-arginine methyl ester (L-NAME) (15 mg/kg, n = 4), both the VEGF mimetic and L-NAME (15 mg/kg, n = 5), Nω-(mimosoyl)-L-lysine (L-NIL) (3 mg/kg, n = 5), or both the VEGF mimetic and L-NIL (n = 5). On day 7, the treated mice were euthanized, and ulcer areas were determined. L-NAME is a nonselective inhibitor of NOS (24); that is, it inhibits all three of the identified isoforms of this enzyme. L-NIL is a selective inhibitor for the inducible isoform of NOS (21).

Materials. Indomethacin was purchased from Sigma Chemicals (St. Louis, MO) and was suspended in 1% carboxymethylcellulose sodium. Lumiracoxib was purchased from SynphaBase AG (Muttenz, Switzerland) and was suspended in 1% carboxymethylcellulose cellulose sodium. L-NAME (Sigma) and L-NIL (Sigma) were dissolved in 0.9% saline. VEGF was purchased from Chemicon International (Temecula, CA) and was dissolved in sterile distilled water.

Statistical analysis. All data are expressed as the means ± SE. Comparisons of data among multiple groups were performed with one-way ANOVA followed by Dunnett’s multiple comparison test. Comparisons between two groups were conducted using the unpaired Student’s t-test. An associated probability of less than 5% was considered significant.

RESULTS
Enhancement of gastric ulcer healing by a VEGF mimetic. Gastric ulcers were clearly visible and of relatively consistent size 3 days after application of acetic acid to the serosal surface of the mouse stomach. The ulcers penetrated into the submucosal layer. In mice treated twice daily with vehicle, the size of the ulcers decreased over time (Fig. 1). A significantly more pronounced reduction of ulcer area was observed in mice treated twice daily with the VEGF mimetic.

The VEGF mimetic accelerated ulcer healing in a dose-dependent manner (Fig. 2). Intraperitoneal treatment at a dose of 25 ng/kg did not significantly affect gastric ulcer healing compared with that in mice treated with vehicle. However, at doses of 50 and 100 ng/kg, the VEGF mimetic significantly enhanced ulcer healing.

The VEGF mimetic also significantly enhanced ulcer healing when given orally (Fig. 3). Thus oral treatment with the VEGF mimetic from days 3 through 7 after induction of ulcers resulted in significantly smaller ulcers compared with treatment orally with vehicle that were not significantly different than what was observed in mice treated intraperitoneally with the VEGF mimetic.

In contrast to the improved healing observed in mice treated with the VEGF mimetic, treatment with the full-length VEGF protein did not significantly affect ulcer healing when given either intraperitoneally or orally (Fig. 3). Also, intraperitoneal treatment with a peptide with the reverse sequence of the VEGF mimetic did not significantly affect ulcer healing compared with vehicle treatment (8.6 ± 0.8 vs. 10.8 ± 1.4 mm², respectively; n = 6–7).

As in mice, the ulcers in rats were well established 3 days after application of acetic acid to the serosal surface of the stomach. The mean ulcer area at day 3 was 118 ± 6 mm². By day 7, the mean ulcer area in rats treated with vehicle had declined to 91 ± 10 mm², but a significantly greater reduction of ulcer area was observed in the rats treated intraperitoneally with 100 ng/kg of the VEGF mimetic (35 ± 15 mm²; P < 0.05 vs. the vehicle-treated group; n = 4 per group).

Lack of effect of the VEGF mimetic on acid secretion. Intraperitoneal administration of the VEGF mimetic at a dose (100 ng/kg) that had a significant effect on ulcer healing did not have any detectable effect on gastric acid secretion. The
volume of gastric juice recovered from the pylorus-ligated stomach 3 h after administration of the VEGF mimetic did not differ significantly from that collected from vehicle-treated rats (0.62 ± 0.09 vs. 0.66 ± 0.06 ml, respectively). The titratable acidity in the gastric juice was also similar in the VEGF mimetic-treated vs. vehicle-treated mice (10.4 ± 2.7 vs. 10.1 ± 1.3 meq, respectively).

Effects of the VEGF mimetic on ulcer healing are COX-2 independent. As in the experiments described above, mice treated with the VEGF mimetic (50 mg/kg) from day 3 to day 7 had an average ulcer area that was significantly smaller than that in the vehicle-treated mice (Fig. 4). Treatment with lumiracoxib, a selective inhibitor of COX-2, did not affect ulcer healing compared with vehicle-treated mice. Moreover, mice treated with the combination of lumiracoxib and the VEGF mimetic still exhibited a significant improvement in the extent of healing compared with vehicle-treated mice.

Effects of the VEGF mimetic on ulcer healing are NO dependent. Treatment with L-NAME did not significantly affect ulcer healing (Fig. 5). However, when L-NAME was coadministered with the VEGF mimetic, ulcer healing occurred to a similar extent as was observed with vehicle treatment; that is, a significant effect of the VEGF mimetic on ulcer healing was no longer apparent.

To determine whether selective inhibition of iNOS would produce the same inhibitory effect on VEGF-induced enhancement of ulcer healing, the effects of treatment with L-NIL were examined. As was the case with L-NAME, treatment with L-NIL did not influence ulcer healing compared with vehicle (Fig. 5). However, mice cotreated with L-NIL and the VEGF mimetic did not exhibit the extent of ulcer healing that was observed in mice treated with the VEGF mimetic alone.

Effects of the VEGF mimetic on angiogenesis. The effects of the VEGF mimetic on angiogenesis were examined by quantifying the number of microvessels in the ulcer bed (granulation tissue) and in the ulcer margin. Angiogenic microvessels were clearly identifiable in the ulcer bed and ulcer margin after staining for CD31 (Fig. 6). Little if any staining for CD31 was detected in gastric tissue from healthy mice (i.e., no ulcers). The number of microvessels in the ulcer margin did not change significantly between days 3 and 5 after ulcer induction, but there was a significant increase in angiogenesis in the granulation tissue (Figs. 6 and 7). In mice treated with the VEGF mimetic, the extent of angiogenesis was similar to that in the vehicle-treated group.

Contrary to our hypothesis, the beneficial effects of the VEGF mimetic on ulcer healing did not appear to be due to enhancement of angiogenesis. Reconstruction of the destroyed vascular network within the ulcerated site is integral to healing because granulation tissue formation and tissue deposition are dependent upon nutrient availability and nutrients are delivered to the injured site via capillaries. As observed previously (15, 16), there was a marked increase in microvessel density in response to the induction of an ulcer in the stomach. However, treatment with the VEGF mimetic did not increase the extent of angiogenesis above that observed in vehicle-treated rats. Our hypothesis that the VEGF mimetic would enhance angiogenesis was based on the fact that the mimetic was designed to replicate the region of the VEGF protein responsible for binding to and activating the VEGF receptors. This pentadecapeptide significantly accelerated gastric ulcer healing in mice and rats. Significant effects were observed with a dose as low as 50 ng/kg and when given orally or intraperitoneally. The effect of the mimetic was specific, in that the reverse-sequence pentadecapeptide did not significantly affect ulcer healing. Moreover, an equimolar dose of VEGF did not affect ulcer healing, whether given orally or intraperitoneally.

DISCUSSION

VEGF is the most potent known stimulator of angiogenesis (31), a process integral to all types of wound healing. In the present study, we examined the effects on gastric ulcer healing of a VEGF mimetic modeled on a portion of the VEGF region responsible for binding to and activating the VEGF receptors. This pentadecapeptide significantly accelerated gastric ulcer healing in mice and rats. Significant effects were observed with a dose as low as 50 ng/kg and when given orally or intraperitoneally. The effect of the mimetic was specific, in that the reverse-sequence pentadecapeptide did not significantly affect ulcer healing. Moreover, an equimolar dose of VEGF did not affect ulcer healing, whether given orally or intraperitoneally.

In considering other potential mechanisms of action of the VEGF mimetic, we first considered the possibility that the...
mimetic might reduce gastric acid secretion since inhibitors of acid secretion are the mainstay of treatment of gastric ulcers and have been shown to be effective in this model (9). However, the mimetic, when administered at a dose that was effective in enhancing ulcer healing, had no effect on the volume of gastric juice or its acidity.

We then examined the possibility that the beneficial effects of the VEGF mimetic were mediated via COX-2. COX-2 plays an important role in gastric mucosal defense (43) and can be rapidly upregulated in response to mucosal irritants, including aspirin (6). Prostaglandins produced in the ulcerated mouse stomach are largely derived from COX-2 (19), and inhibition of COX-2 activity has been shown to significantly delay ulcer healing (15, 20, 28). Moreover, treatment with conventional nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors at doses that delay ulcer healing in rats was also found to alter the ratios of pro- and antiangiogenic factors (VEGF and endostatin, respectively) in serum. COX-2-derived prostaglandins can stimulate the release of VEGF (19), whereas NSAIDs can interfere with downstream proangiogenic signaling of VEGF (30). In the present study, we found that treatment with a selective COX-2 inhibitor (lumiracoxib) did not interfere with the beneficial effects of the VEGF mimetic on ulcer healing in mice. Importantly, we confirmed that the dose of lumiracoxib used in these experiments produced a significant (>90%) inhibition of COX-2 activity in the mouse.

Like prostaglandins, NO contributes significantly to gastric mucosal defense and healing (42, 44). Inhibitors of NO synthesis have been shown to delay ulcer healing in rodent models (12, 14), while NO donors can significantly accelerate healing in these models (7, 12, 15). Expression of iNOS is apparent in inflammatory cells in the ulcer bed in rats (14). The delay in gastric ulcer healing that occurs following administration of inhibitors of NO synthesis has been associated with diminished angiogenesis and impaired blood flow at the ulcer margin (3, 12, 35). In the present study, the potential role of NO synthesis in the actions of the VEGF mimetic was examined through the use of a nonselective NOS inhibitor (l-NAME) and a selective iNOS inhibitor (l-NIL). Neither of these inhibitors, alone, significantly affected ulcer healing when administered over a period of 4 days. However, both inhibitors interfered with the ability of the VEGF mimetic to enhance ulcer healing. Moreover, inhibition of iNOS activity resulted in a significant reduction of angiogenesis in the ulcer bed and ulcer margin of

Fig. 6. Immunohistochemical staining of the endothelial cell marker CD31 in granulation tissue (A–C) and the ulcer margin (D–F). A and D show day 3 mice, B and E show day 5 vehicle-treated mice (ip), and C and F show day 5 VEGF mimetic-treated mice (50 ng/kg ip).
mice treated with the VEGF mimetic to levels even lower than those observed in vehicle-treated rats. This is consistent with the findings of previous studies that NO is also crucial for VEGF-dependent angiogenesis (22, 47), but, to our knowledge, this is the first demonstration of a beneficial effect on healing being mediated by iNOS.

Gastrointestinal ulceration and bleeding remain significant clinical concerns, particularly in patients taking NSAIDs on a chronic basis (39). Cotherapy with proton pump inhibitors is the most common approach to preventing ulceration and promoting the healing of existing ulcers in these patients. However, there is still a need for agents that will promote more rapid and effective healing. In terms of therapeutic utility, it is noteworthy that the VEGF mimetic was as effective in promoting ulcer healing when given orally as when given systemically. This is in contrast to the experiments in the present study and in a previous study (40), showing that oral (or systemic) administration of the full-length VEGF protein did not affect ulcer healing. Of course, it is possible that higher doses of the full-length VEGF protein would accelerate ulcer healing, but in the present study we tested a dose equimolar to the vehicletreated group; *P < 0.05 vs. the vehicle-treated group; **P < 0.05 vs. the group treated only with the VEGF mimetic. There were no statistical differences between the vehicle and the VEGF mimetic-treated groups.

In summary, the studies described herein demonstrate that a 15-amino acid mimetic of VEGF is a very potent stimulus for gastric ulcer healing in rodents and is active when given orally or systemically. The acceleration of healing by this mimetic is dependent upon iNOS activity but not on COX-2 activity. From the present studies, it would appear that the acceleration of ulcer healing induced by the VEGF mimetic is not related to an enhancement of angiogenesis in the ulcerated tissue. Given its oral bioavailability and potency, this mimetic may have therapeutic utility for the treatment of ulceration in the gastrointestinal tract.

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REFERENCES


