Platelets modulate gastric ulcer healing: Role of endostatin and vascular endothelial growth factor release

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Contributed by Louis J. Ignarro, March 26, 2001

Bleeding and delayed healing of ulcers are well recognized clinical problems associated with the use of aspirin and other nonsteroidal antiinflammatory drugs, which have been attributed to their antiaggregatory effects on platelets. We hypothesized that antiplatelet drugs might interfere with gastric ulcer healing by suppressing the release of growth factors, such as vascular endothelial growth factor (VEGF), from platelets. Gastric ulcers were induced in rats by serosal application of acetic acid. Daily oral treatment with vehicle, aspirin, or ticlopidine (an ADP receptor antagonist) was started 3 days later and continued for 1 week. Ulcer induction resulted in a significant increase in serum levels of VEGF and a significant decrease in serum levels of endostatin (an antiangiogenic factor). Although both aspirin and ticlopidine markedly suppressed platelet aggregation, only ticlopidine impaired gastric ulcer healing and angiogenesis as well as reversing the ulcer-associated changes in serum levels of VEGF and endostatin. The effects of ticlopidine on ulcer healing and angiogenesis were mimicked by immunodepletion of circulating platelets, and ticlopidine did not influence ulcer healing when given to thrombocytopenic rats. Incubation of human umbilical vein endothelial cells with serum from ticlopidine-treated rats significantly reduced proliferation and increased apoptosis, effects reversed by an antibody directed against endostatin. Ticlopidine treatment resulted in increased platelet endostatin content and release. These results demonstrate a previously unrecognized contribution of platelets to the regulation of gastric ulcer healing. Such effects likely are mediated through the release from platelets of endostatin and possibly VEGF. As shown with ticlopidine, drugs that influence gastric ulcer healing may do so in part through altering the ability of platelets to release growth factors.

angiogenesis | ticlopidine | aspirin | proliferation | endothelium

In addition to topical irritation of the gastric mucosa, aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) cause gastric damage through inhibition of prostaglandin synthesis and by producing microcirculatory injury (1). Most NSAIDs, particularly aspirin, are potent inhibitors of platelet function. NSAIDs also delay ulcer healing (2), at least in part through inhibition of cyclooxygenase-2 (3). Although inhibitory effects of NSAIDs on platelet aggregation appear likely to contribute to the bleeding of ulcers, the contribution of antiplatelet effects of these drugs in terms of inhibition of ulcer healing has not been reported previously.

Angiogenesis is a pivotal process in all types of wound healing, including the healing of gastric ulcers (3, 4). It is regulated by proangiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor, and EGF, and by antiangiogenic factors, such as endostatin. Platelet aggregation is a cardinal feature of vascular repair. A variety of potent angiogenic stimulators, including VEGF (5), platelet-derived endothelial cell growth factor (6), EGF (7), and platelet-derived growth factor (8), are stored in platelets and released during clotting at the wound. Activation of platelets by α-thrombin (9) stimulates angiogenesis in the chick chorioallantoic membrane (10). Furthermore, platelets have been shown to stimulate endothelial cell proliferation and capillary tubular structure formation *in vitro* (11).

Of the many growth factors, VEGF is the most potent stimulus for angiogenesis (12), whereas endostatin is an extremely potent inhibitor of angiogenesis (13). Endostatin inhibits endothelial cell proliferation (13) and migration (14) as well as inducing apoptosis (15). However, whether or not endostatin is contained within platelets is not known.

The purpose of the present study was to determine whether inhibition of platelet aggregation *per se* would result in a delay of gastric ulcer healing in the rat. Two antiplatelet drugs were used: aspirin and ticlopidine. Ticlopidine inhibits platelet aggregation by blocking the interaction of ADP with its receptor, whereas aspirin blocks platelet aggregation through inhibition of thromboxane synthesis. We then evaluated the possibility that effects of these antiplatelet drugs other than inhibition of aggregation might influence ulcer healing. Specifically, effects on the release of pro- and antiangiogenic growth factors (VEGF and endostatin, respectively) from platelets were examined. The results demonstrate that platelets play a pivotal role in modulating gastric ulcer healing, most likely through the release of growth factors such as VEGF and endostatin. Ticlopidine can influence angiogenesis, a critical component of ulcer healing, through modulation of the release of these growth factors from platelets. These effects of ticlopidine were produced independent of its effects on platelet aggregation.

Materials and Methods

Animals. All experiments were approved by the University of Calgary Animal Care Committee. Male Wistar rats (175–200 g) were fed standard laboratory chow and tap water and were kept in a room with controlled temperature (22 ± 1°C), humidity (65–70%), and light cycle (12-h light/12-h dark).

Effects of Antiplatelet Drugs on Gastric Ulcer Healing. The rats were fasted for 18 h. Under halothane anesthesia, acetic acid (0.5 ml, 80% vol/vol) was applied to the serosal surface of the stomach for 1 min via a 3-ml syringe barrel (16, 17). The abdomen was sutured closed and the rats were returned to their cages. Rats

Abbreviations: NSAID, nonsteroidal antiinflammatory drug; VEGF, vascular endothelial growth factor; EGF, endothelial growth factor; PPP, platelet-poor plasma; HUVECs, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6′-diamidino-2-phenylindole.

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 www.pnas.org/cgi/doi/10.1073/pnas.111150798

were given vehicle (0.5% carboxymethycellulose; 5 ml/kg), ticlopidine (100 or 300 mg/kg), or aspirin (30 mg/kg) intragastrically each day from day 3 to day 9 postulcer induction. These doses of ticlopidine and aspirin have been shown to inhibit platelet aggregation and thrombus formation in the rat (18–23). Aspirin's inhibitory effects on thromboxane (TX) synthesis were confirmed by taking blood before sacrifice, allowing it to clot at 37°C for 45 min, and then measuring TXB_2 levels by ELISA (20).

Other groups of rats ($n = 5$ each) received i.v. ticlopidine (30 mg/kg) or vehicle daily from day 3 to day 9 postulcer induction, and the ulcer area was determined on day 10. For i.v. administration (via a tail vein), ticlopidine was dissolved in 0.9% sterile saline and administered in a volume of 5 ml/kg body weight as a slow infusion.

The effects of immunodepletion of platelets on gastric ulcer healing also was examined. Rats in which ulcers had been induced (as above) were given antiplatelet serum ($n = 9$) or normal rabbit serum ($n = 7$) i.v. on days 3 and 7, as described previously (24). The rats were killed on day 10 for assessment of ulcer area. Treatment with this antiplatelet serum reduces the numbers of circulating platelets in the rat by >99% (24). The antibody had no effect on circulating numbers of erythrocytes or leukocytes. In other experiments, groups of rats ($n = 6$ each) made thrombocytopenic, as above, were treated with ticlopidine (300 mg/kg per day) or vehicle for 1 week to determine effects on ulcer healing. Other thrombocytopenic rats (with gastric ulcers) were given washed platelets harvested from rats that had been treated for 1 week with ticlopidine (300 mg/kg) or vehicle. The platelets (10^9/rat) were infused i.v. on the fourth day after ulcer induction, and the ulcer area was measured on day 10.

Assessment of Ulcer Healing. On day 10 after ulcer induction, rats were anesthetized with halothane and a blood sample was drawn from the inferior vena cava for measurement of hematocrit. The stomach then was removed and the ulcer area was measured planimetrically (17) in a blind manner. A longitudinal section of tissue that included the ulcer base and both sides of ulcer margins was fixed in 4% neutral buffered formalin (4°C) and then embedded in paraffin and sectioned.

Assessments of Angiogenesis. Angiogenesis was assessed by counting the number of neomicrovessels, using immunohistochemistry for von Willebrand Factor (25). Three randomly selected areas of the granulation tissue on each slide were counted and the data were averaged. Any positive-staining endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered an angiogenic microvessel (26).

Platelet Aggregation. Rats were given vehicle, ticlopidine (300 mg/kg), or aspirin (30 mg/kg) orally once daily for 7 days. Blood was collected from the descending aorta 3 h after the final dose, and platelet-rich plasma and platelet-poor plasma (PPP) were prepared (20). The effects of aspirin and ticlopidine on platelet aggregation were studied *ex vivo* (20). Three agonists were used: thrombin (0.375–1.25 units/ml), ADP (2.5–20 μM), and arachidonic acid (20–750 μg/ml).

Measurement of VEGF and Endostatin. Platelet-rich plasma was stimulated with thrombin (0.75 unit/ml) or ADP (10 μM) in the cuvette of a platelet aggregometer. The platelet suspension then was centrifuged (9,000 $\times$ *g*), and the supernatant was stored at −70°C. The concentrations of VEGF and endostatin were measured by ELISA. In some experiments, the levels of VEGF and endostatin in PPP and in lysed platelets also were measured.

Culture of Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Manassas, VA) and maintained in modified F12K medium supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, and 10% FBS. They were used at passages 38–44 (26).

Endothelial Cell Proliferation. Cell proliferation was determined by using the MTT [3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyltetrazolium bromide] assay (27). HUVECs (2×10^4/well) were incubated with F12K medium with 2% FBS in 24-well plates. Beginning 4 h after plating, the cells were incubated for 24 h with serum from rats that had been treated once daily for the previous week with vehicle, ticlopidine (300 mg/kg), or aspirin (30 mg/kg) or directly with ticlopidine (1–10 μM) or aspirin (1–10 μM). The medium then was aspirated and MTT was added to each well (0.25 mg/ml). The cells then were incubated for a further 3 h at 37°C. The medium then was aspirated and the cells were lysed with DMSO. An aliquot of the lysate was transferred to a 96-well plate, and absorbance at 540 nm was measured. For cell counts, cells were detached by trypsin and diluted with PBS. The number of cells in each well was counted under a light microscope.

Apoptosis. Apoptosis, defined as nuclear chromatin condensation and fragmentation, was assessed blindly after staining with DAPI (4′,6′-diamidino-2-phenylindole; 1 μg/ml) (28). Apoptosis also was measured with a commercially available ELISA assay that quantifies DNA fragmentation, with the results expressed as a percentage of medium control values (cells incubated with 2.5% FBS).

Statistical Analysis. All data are expressed as mean ± SEM, with sample sizes of at least five per group. Comparisons of data among groups were performed with one-way ANOVA followed by the Student–Newman–Keuls test. An associated probability (*P* value) of less than 5% was considered significant.

Materials. Reagents were obtained from the following sources: ticlopidine, aspirin, heparin, endothelial cell growth supplement and DAPI were from Sigma; antibodies and ELISA kits for measurement of VEGF and endostatin were from Chemicon; apoptosis ELISA kits were from Boehringer Mannheim; HUVECs and medium were from American Type Culture Collection; antiplatelet serum was from Cedarlane Laboratories; and all other supplies were from Fisher Scientific.

Results

Gastric Ulcer Healing and Angiogenesis. The mean size of ulcers at day 3 after induction was 78 ± 8 mm^2. In the days that followed, granulation tissue began to develop and microvessels grew (angiogenesis) from the adjacent normal tissue. By day 10, ulcers in the control group had healed significantly (by an average of almost 80%; Fig. 1*A*). Treatment with the higher dose of ticlopidine reduced the extent of gastric ulcer healing by ≈50% (Fig. 1*A*) and depressed angiogenesis in the granulation tissue (Fig. 1*B*). Aspirin and the lower dose of ticlopidine were without significant effects on ulcer healing. None of the treatments significantly affected the hematocrit, which was measured on day 10 after ulcer induction. Aspirin treatment for 1 week reduced whole-blood TXB_2 synthesis by >99% (from 1,867 ± 113 ng/ml to 13 ± 5 ng/ml; $P < 0.001$).

Intravenous administration of ticlopidine (30 mg/kg) daily for a week also significantly delayed gastric ulcer healing (mean ulcer area of 55.1 ± 5.0 mm^2 vs. 23.4 ± 1.9 mm^2 in vehicle-treated rats; $P < 0.01$).

Induction of thrombocytopenia with antiplatelet serum caused a significant inhibition of gastric ulcer healing (mean ulcer size of 38.0 ± 5.6 mm^2 vs. 17.9 ± 2.8 mm^2 in rats treated with normal rabbit serum; $P < 0.05$), a significant decrease in numbers of angiogenic microvessels (61.4 ± 4.7 vs. 86.0 ± 6.3 per

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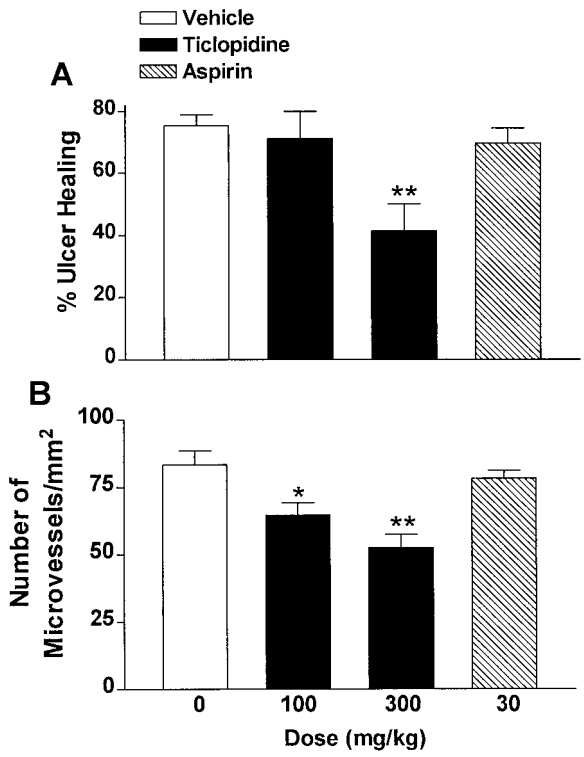

Fig. 1. Effects of ticlopidine and aspirin on gastric ulcer healing (*A*) and angiogenesis in the granulation tissue (*B*) 10 days after ulcer induction. Three days after ulcer induction, the mean ulcer size was 78 ± 8 mm^2. The rats were given vehicle, ticlopidine (100 or 300 mg/kg), or aspirin (30 mg/kg) orally each day from day 3 to day 9 postulcer induction. Ulcer healing is expressed as a percent reduction in ulcer size from that on day 3. *, $P < 0.05$; **, $P < 0.01$ vs. the corresponding vehicle-treated group (the statistical analysis was performed by using the raw ulcer area data).

mm^2, respectively; $P < 0.01$), but no significant effect on hematocrit (37.9 ± 0.6 vs. 39.3 ± 0.4, respectively). Daily administration of ticlopidine (300 mg/kg per day) to thrombocytopenic rats did not significantly affect ulcer healing relative to that in thrombocytopenic rats treated with vehicle (mean ulcer areas of 34.9 ± 5.2 mm^2 vs. 40.7 ± 5.6 mm^2, respectively). However, infusion of washed platelets from rats treated for 1 week with ticlopidine into thrombocytopenic rats resulted in a significant inhibition of ulcer healing relative to thrombocytopenic rats given platelets from rats treated with vehicle (25.6 ± 5.6 mm^2 vs. 11.1 ± 3.0 mm^2, respectively; $P < 0.05$).

Platelet Aggregation. Platelet counts were not affected significantly by treatment with ticlopidine or aspirin. Platelets from ticlopidine- and aspirin-treated rats showed a similar, low degree of aggregation in response to thrombin and arachidonic acid compared with those from vehicle-treated rats (Fig. 2 *A* and *B*). However, platelets from ticlopidine-treated rats exhibited significantly lower aggregation induced by ADP than those from aspirin- or vehicle-treated rats (Fig. 2*C*).

Serum VEGF and Endostatin Levels. In rats with gastric ulcers, serum levels of VEGF were significantly elevated (Fig. 3*A*), whereas serum levels of endostatin were markedly decreased (Fig. 3*B*). Treatment with the higher dose of ticlopidine prevented the elevation of serum VEGF and the decrease in serum endostatin (Fig. 3). Aspirin and the lower dose of ticlopidine (100 mg/kg) had no effect on the serum levels of VEGF or endostatin.

Ticlopidine treatment (300 mg/kg per day) of rats that did not have ulcers resulted in a modest increase in serum endostatin levels (vehicle, 16.7 ± 0.4 ng/ml; ticlopidine, 18.2 ± 0.3 ng/ml; $P < 0.01$), but did not significantly affect serum VEGF levels (vehicle, 0.76 ± 0.09 ng/ml; ticlopidine, 0.80 ± 0.04 ng/ml).

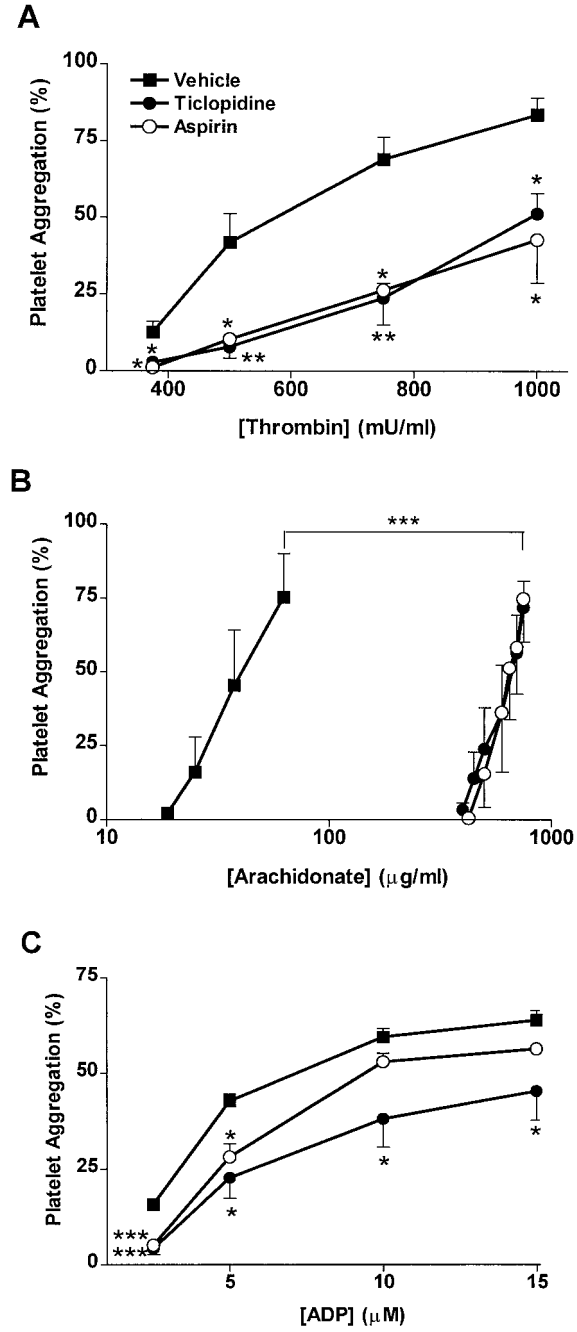

Fig. 2. Platelet aggregation induced by thrombin (*A*), arachidonic acid (*B*), and ADP (*C*). Rats were given vehicle, ticlopidine (300 mg/kg), or aspirin (30 mg/kg) orally each day for 7 days. Three hours after the final dose, platelet-rich plasma was prepared and platelet aggregation in response to the three agonists was assessed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. the corresponding control group.

HUVEC Proliferation and Apoptosis. Exposure of HUVECs to serum from vehicle-treated rats resulted in a concentration-dependent increase in proliferation (Fig. 4*A*). However, the stimulation of proliferation was significantly less when the cells

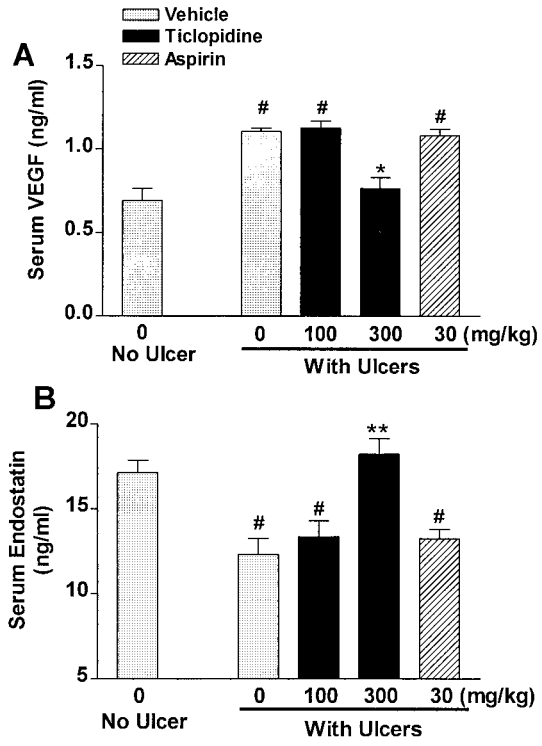

Fig. 3. Effects of ticlopidine and aspirin on serum VEGF (*A*) and endostatin (*B*) levels. Beginning 3 days after ulcer induction, rats were given vehicle, ticlopidine (300 mg/kg), or aspirin (30 mg/kg) orally each day for 7 days. The "No ulcer" group consisted of sham-operated rats that were treated each day with vehicle. *, $P < 0.05$; **, $P < 0.01$ vs. the corresponding vehicle-treated group. #, $P < 0.01$ vs. the "No ulcer" group.

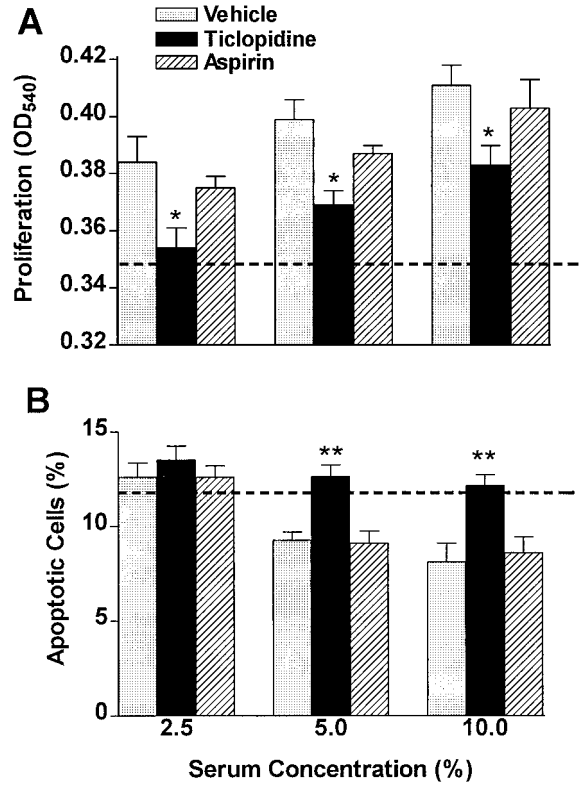

Fig. 4. Effects of serum derived from ticlopidine- or aspirin-treated rats on HUVEC proliferation (*A*) and apoptosis (*B*). HUVECs were incubated with sera from vehicle-treated, ticlopidine-treated (300 mg/kg), or aspirin-treated (30 mg/kg) rats for 24 h. Cell proliferation was determined by the MTT assay, and apoptosis was assessed by DAPI staining (*B*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. the corresponding vehicle-treated group. The dotted lines show the mean levels of proliferation or apoptosis in cells incubated only with the control medium (containing 2.5% FBS, but no rat serum).

were incubated with serum from ticlopidine-treated rats. Serum from aspirin-treated rats increased proliferation to the same extent as serum from vehicle-treated rats. Incubation of HUVECs directly with ticlopidine or aspirin (1–10 μM) did not affect the rate of proliferation. Determining cell proliferation by direct cell counting, rather than with the MTT assay, produced similar results (data not shown).

An average of 12% of HUVECs exposed only to medium were undergoing apoptosis. Serum from vehicle-treated rats reduced apoptosis concentration-dependently. In contrast, serum from ticlopidine-treated rats did not reduce apoptosis. Serum from aspirin-treated rats had no significant effect on apoptosis compared with that from vehicle-treated rats (Fig. 4*B*). Quantification of apoptosis by measurement of DNA fragmentation yielded virtually identical results (data not shown). Direct incubation of HUVECs with ticlopidine or aspirin (1–10 μM) did not affect the rate of apoptosis.

To examine further the possibility that the increased levels of endostatin in serum from ticlopidine-treated rats contributed to the reduced HUVEC proliferation and increased apoptosis, the effects of addition to the culture medium of an antiendostatin antibody (16 μg/ml), an anti-VEGF antibody (2.5 μg/ml), or an isotype-matched control antibody (16 μg/ml) were assessed. These antibodies were the same as those used in the ELISA assays for endostatin and VEGF. Antiendostatin prevented both the decreased proliferation and increased apoptosis associated with exposure of the HUVECs to serum from ticlopidine-treated rats (Fig. 5). However, neither the anti-VEGF nor the control antibody significantly affected the ticlopidine-associated changes in proliferation or apoptosis. The three antibodies had no effect on proliferation or the rate of apoptosis in HUVECs exposed only to control medium (data not shown).

Platelet VEGF and Endostatin. The levels of VEGF in serum, PPP, and platelet lysates, derived from the same volume of blood, were 1.12 ± 0.03, 0.79 ± 0.05, and 1.57 ± 0.13 ng/ml, respectively. This suggests that VEGF is stored within platelets. Ticlopidine did not significantly affect VEGF levels in PPP or in platelet lysates (0.79 ± 0.06 and 1.47 ± 0.08 ng/ml, respectively), but significantly decreased serum VEGF levels (0.82 ± 0.07 ng/ml; $P < 0.05$). Thus, ticlopidine affected the release of VEGF from platelets, not its levels within the platelets. Thrombin and ADP did not significantly affect the *in vitro* release of VEGF from platelets from vehicle-treated rats (Fig. 6*A*). Significant inhibition of ADP-induced VEGF release was observed with platelets derived from ticlopidine-treated rats.

Endostatin levels in serum, PPP, and platelet lysates, derived from the same volume of blood, were 10.7 ± 0.7, 7.9 ± 0.4, and 19.7 ± 1.1 ng/ml, respectively. Ticlopidine treatment significantly increased endostatin levels in all three types of samples (15.8 ± 0.7, 13.7 ± 0.7, and 29.2 ± 2.9 ng/ml, respectively; $P <$ 0.05 for each). This indicates that endostatin is stored within platelets. Stimulation of platelets from vehicle-treated rats with thrombin, but not ADP, resulted in a significant increase in endostatin release (Fig. 6*B*). Measurement of residual endostatin in the platelet pellet revealed that thrombin stimulation resulted in the release of ≈85% of the total endostatin content of the platelet. Both basal- and thrombin-stimulated endostatin

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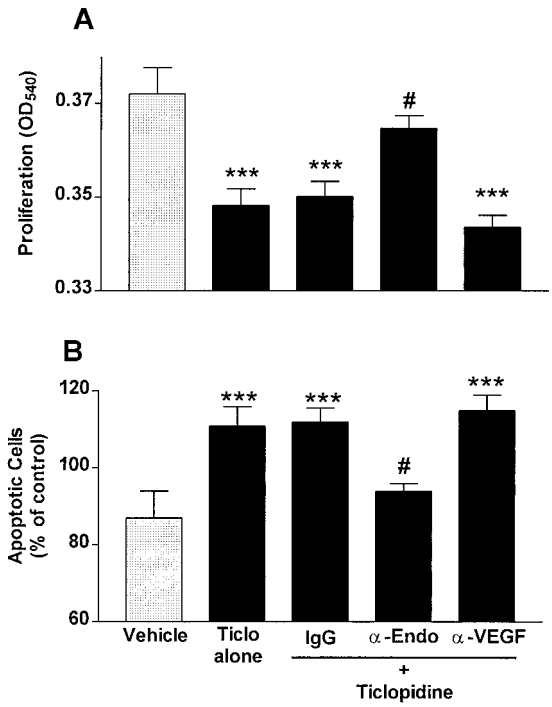

Fig. 5. Effects of antiendostatin, anti-VEGF, and control antiserum (IgG) on HUVEC proliferation (*A*) and apoptosis (*B*; DAPI staining) after exposure to 5% rat serum. Serum from ticlopidine-treated rats reduced proliferation and increased apoptosis (***, $P < 0.001$). Antiendostatin abolished the effects of the serum from ticlopidine-treated rats (#, $P < 0.05$ vs. the "ticlopidine alone" and ticlopidine + IgG groups).

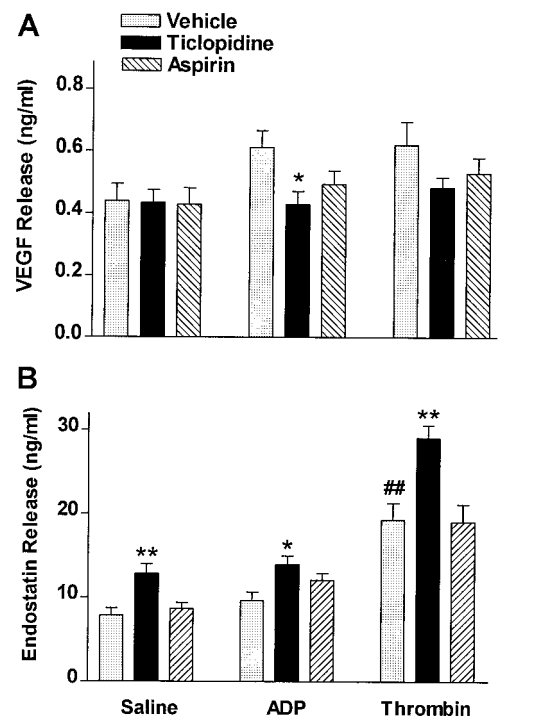

Fig. 6. Release of VEGF (*A*) and endostatin (*B*) from platelets in response to stimulation with ADP or thrombin. Platelets were harvested from rats that had been treated daily for 1 week with vehicle, ticlopidine (300 mg/kg), or aspirin (30 mg/kg) and then were challenged *in vitro* with saline, ADP (10 μM), or thrombin (0.75 unit/ml). *, $P < 0.05$; **, $P < 0.01$ vs. corresponding vehicle-treated control; ##, $P < 0.01$ vs. the corresponding saline-challenged group.

release were enhanced further when the platelets were from ticlopidine-treated rats (Fig. 6*B*). When platelets from rats treated with aspirin were examined, the release of endostatin in response to stimulation with thrombin or ADP was not significantly different from that seen with platelets from vehicle-treated rats.

Discussion

Angiogenesis is an essential process for ulcer healing, requiring the concerted interaction of a variety of cellular systems. Platelets play a pivotal role in wound healing by releasing a number of growth factors that can promote angiogenesis (5, 7, 8, 29). Therefore, we hypothesized that, in addition to inhibitory effects on platelet aggregation, antiplatelet drugs might impair gastric ulcer healing by virtue of inhibitory effects on growth factor release. Our results demonstrate a previously unrecognized role for platelets in gastric ulcer healing in the rat. Moreover, we have demonstrated that platelets contain the antiangiogenic factor endostatin. Treatment with ticlopidine, but not aspirin, significantly delayed ulcer healing and significantly modulated serum levels of endostatin and VEGF, and their release from platelets, in a manner consistent with impairment of ulcer healing. Ticlopidine did not alter gastric ulcer healing in thrombocytopenic rats, but platelets from ticlopidine rats were able to delay ulcer healing when transfused into thrombocytopenic rats, further supporting the hypothesis that the effects of this drug on healing were mediated through its actions on the platelet.

Angiogenesis can be influenced by a variety of factors. In the present study, we focused on one proangiogenic factor (VEGF) and one antiangiogenic factor (endostatin). VEGF is a highly specific mitogen for vascular endothelial cells that promotes endothelial proliferation and migration (30) and has been shown to significantly accelerate ulcer healing (12). Endostatin is one of the most potent, endogenous inhibitors of angiogenesis (13). It directly inhibits endothelial growth and migration, promotes apoptosis, and antagonizes the angiogenesis-promoting effects of VEGF (31). In normal rats, both of these factors were detected in serum. Induction of gastric ulcers resulted in increases in serum levels of VEGF and decreases in serum levels of endostatin. Such changes are consistent with a need for increased angiogenesis in an effort to heal the ulcer. In rats treated orally with ticlopidine, these shifts in serum growth factor levels were abolished, and, in parallel, ticlopidine significantly impaired ulcer healing. Significant impairment of ulcer healing also was observed when ticlopidine was given i.v. at 1/10 the oral dose. It is unlikely that the platelet was the only source of VEGF and endostatin in the serum. However, the notion that ticlopidine may have impaired ulcer healing through inhibition of platelet release of these growth factors is supported by the demonstration that depletion of circulating platelets with an antiplatelet serum produced an impairment of ulcer healing and a reduction in angiogenesis in the ulcer bed similar in magnitude to that seen with ticlopidine treatment.

VEGF is stored in α-granules within the platelet (29). Serum VEGF levels previously have been shown to correlate with the numbers of circulating platelets (32). Given that ticlopidine inhibits ADP-induced platelet aggregation (33) and the resulting α-granule release (34), it is predictable that VEGF release from platelets would be reduced after ticlopidine treatment. On the other hand, the increased release of endostatin from platelets harvested from ticlopidine-treated rats occurred despite the fact that ticlopidine markedly suppressed platelet aggregation. Thus, endostatin release occurs independently of platelet aggregation. The studies of platelet lysates demonstrated that ticlopidine treatment resulted in an increase of platelet endostatin content

of ≈50%. It is interesting that substantial endostatin release was stimulated by thrombin, with little or no effect of ADP, despite the fact that these two agonists, at the doses used, stimulated platelet aggregation to approximately the same extent (60–70% of maximal aggregation).

The addition of rat serum to cultured HUVECs caused a concentration-dependent increase in proliferation and a decrease in apoptosis. However, the serum from rats that had been treated with ticlopidine had a greatly reduced capacity to produce these effects. Extending these findings to the *in vivo* situation would suggest that ticlopidine impairs angiogenesis, which is consistent with the observed inhibitory effects of this drug on ulcer healing. Such effects of ticlopidine are due to the enhanced release of endostatin, because the effects on endothelial cell proliferation and apoptosis were reversed by an antiendostatin antibody.

Ticlopidine and aspirin inhibited platelet aggregation to a similar extent, but unlike ticlopidine, aspirin did not impair ulcer healing at the dose tested. Thus, inhibition of platelet aggregation *per se* is not sufficient to interfere with ulcer healing. That aspirin did not significantly affect serum levels of VEGF and endostatin and did not affect endothelial proliferation or apoptosis are likely reasons for the lack of impairment of ulcer healing in rats treated with this drug. Of course, it is possible that other NSAIDs or higher doses of aspirin might influence the release of VEGF and endostatin from platelets and delay ulcer healing. We have observed previously that daily treatment with diclofenac over a period of 1 week significantly impaired ulcer healing and reduced hematocrit in the same model as used in the present study (17). Whether or not diclofenac influenced serum or platelet levels of VEGF and endostatin was not examined in that study. Of course, it is possible that NSAIDs and selective COX-2 inhibitors produce inhibitory effects on ulcer healing through mechanisms other than modulation of platelet growth factor release. These agents have been shown to promote apoptosis and inhibit angiogenesis through direct effects on endothelial cells *in vitro* (3).

Ticlopidine was used in the present study as a pharmacological tool, with the higher dose selected because it is effective in terms of inhibiting platelet aggregation in the rat (19, 22, 23). Further studies to evaluate the effects of therapeutic doses of ticlopidine on the production of angiogenesis-related growth factors in humans seem warranted. Interestingly, inhibitory effects of ticlopidine on proliferation and migration of human saphenous endothelial cells (an *in vitro* model of wound healing) have been reported (35).

In conclusion, we report the previously unrecognized ability of platelets to modulate gastric ulcer healing. Moreover, platelets contain the antiangiogenic factor endostatin, and its release from platelets can be modulated by treatment with ticlopidine. Results of *in vitro* studies of endothelial cell proliferation and apoptosis are consistent with the hypothesis that it is the increased release of antiangiogenic (endostatin) factors and decreased release of proangiogenic factors from sources such as the platelet that account for the modulation of gastric ulcer healing.

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR). L.M. is supported by a Fellowship from AstraZeneca/Canadian Association of Gastroenterology/CIHR. J.L.W. is a CIHR Senior Scientist and an Alberta Heritage Foundation for Medical Research Senior Scientist.

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