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Platelets accelerate gastric ulcer healing through presentation of vascular endothelial growth factor

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1 Platelets contain an array of growth factors that can modulate healing processes, including both pro- (e.g., vascular endothelial growth factor (VEGF)) and antiangiogenic (e.g., endostatin) factors. Previous studies have shown that circulating platelets contribute significantly to gastric ulcer healing, acting as a delivery system for these growth factors to the site of injury. In this study, we examined the effects of orally administered human platelets on the healing of gastric ulcers in rats, and determined the contribution of VEGF and endostatin to healing in this model.

2 Twice-daily administration of human platelets significantly accelerated ulcer healing, but platelet-poor plasma (PPP), lysed platelets and serum failed to produce this effect. There was no correlation between ulcer healing and the levels of VEGF or endostatin in serum, PPP or platelet-rich plasma (PRP).

3 Accelerated ulcer healing could not be produced by oral administration of the angiogenic factors themselves, at concentrations matching those in PRP.

4 The accelerated healing induced by platelets could be reversed by immuno-neutralization of VEGF. In contrast, immuno-neutralization of endostatin did not affect PRP-induced ulcer healing.

5 These studies indicate that VEGF released from platelets accounts for the accelerated healing of gastric ulcers. However, as intact (rather than lysed) platelets were required for the accelerated healing, the presentation of VEGF by the platelet at the site of injury appears to be crucial for enhancement of the healing process.
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Abbreviations: NSAID, nonsteroidal anti-inflammatory drug; PPP, platelet-poor plasma; PRP, platelet-rich plasma; VEGF, vascular endothelial growth factor

Introduction

Platelets contain a wide range of factors capable of promoting tissue growth and new blood vessel formation (angiogenesis), including vascular endothelial growth factor (VEGF), transforming growth factor-β and platelet factor-4 (Linder *et al*., 1979; Maloney *et al*., 1998; Von Hundelshausen *et al*., 2001). They also contain factors that can impair angiogenesis and healing, such as endostatin (Ma *et al*., 2001a, b; 2002; 2005). As platelets accumulate at sites of injury, they can act as a delivery system for these factors. The ability of platelets to promote healing has been exploited in recent years in periodontal regenerative therapy. Administration of platelet-rich plasma (PRP) has been shown to improve results in a variety of procedures in the field of oral and maxillofacial surgery (Carlson & Roach, 2002). PRP has also been found to be effective in the treatment of leg ulcers (Weed *et al*., 2004), and to provide significant benefits in orthopedic, ophthalmic and cosmetic surgery (Anitua *et al*., 2004). The ability of PRP to accelerate healing may be extendable to other types of injuries. In the context of gastric ulcers, we have demonstrated that rats immunodepleted of their platelets exhibit impaired healing, whereas normal rates of healing can be restored through transfusion of platelets from donor rats (Ma *et al*., 2001a).

As platelets contain factors that can accelerate or delay wound healing, the ability of platelets to modulate healing may be regulated by local factors that trigger release of specific growth factors, and/or by the relative content of one growth factor *versus* others. For example, protease-activated receptors (PARs) play an important role in regulating the release from platelets of key pro- and antiangiogenic factors (VEGF and endostatin, respectively) (Ma *et al*., 2005). Thus, activation of PAR1 leads to increased release of VEGF and decreased release of endostatin. Activation of PAR4 has the opposite effect. The relative content of these factors within platelets can also be modulated. Indeed, some drugs that can delay ulcer healing may do so through producing changes in platelet and/or serum levels of pro- *versus* antiangiogenic factors. For example, treatment of rats with ticlopidine or with an NSAID (flurbiprofen or celecoxib) significantly impaired ulcer healing and caused a decrease in the ratio of serum VEGF to endostatin (Ma *et al*., 2001a; 2002). These drugs also affected the release of growth factors from platelets (Ma *et al*., 2001a; 2002).

In the present study, we have further evaluated the role of the platelet as a delivery system for growth factors by testing the hypothesis that orally administered human PRP can

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enhance the healing of gastric ulcers in rats. We then examined the contribution of VEGF and endostatin to the ability of PRP to promote ulcer healing. These studies suggest a crucial role for VEGF in promoting healing, but only in the context of platelet presentation of VEGF.

Methods

Ulcer induction

All experiments were approved by the University of Calgary Animal Care Committee and performed in accordance with the guidelines of the Canadian Council on Animal Care. Male Wistar rats (175–200 g) were fed standard laboratory chow and tap water and were kept in a room with controlled temperature ($22 \pm 1^{\circ}$C), humidity (65–70%), and light cycle (12 h light–12 h dark). The rats were starved for 18 h. Gastric ulcers were induced by serosal application of acetic acid (0.5 ml, 80%) under Halothane anaesthesia, as described (Ma *et al*., 2001a). In other rats, we induced ulceration of the colon through intracolonic administration of 30 mg of trinitrobenzene sulphonic acid (TNBS) in 0.5 ml of ethanol, as described (Morris *et al*., 1989; Wallace *et al*., 1989). Control rats received 0.5 ml of 0.9% saline intracolonically. The rats were anaesthetized with Halothane 72 h later and blood was drawn from the descending aorta for preparation of serum.

Preparation of PRP, plasma and serum

Human blood was taken from healthy volunteers (five male, four female) with 3.4% sodium citrate (8 : 1 vol–vol). The volunteers denied ingesting aspirin or other NSAIDs for at least 14 days before blood collection. The blood was centrifuged at $200 \times g$ for 15 min at room temperature. The PRP was then removed by aspiration. Some of the PRP was further centrifuged at $400 \times g$ for 10 min at room temperature to obtain platelet-poor plasma (PPP). The number of platelets in the PRP was counted by using a haemocytometer and adjusted to 3×10^8 ml^{-1} with PPP. Lysed PRP was prepared by sonicating PRP with a Fisher Model 100 Sonic Dismembrator (Ottawa, Canada) for 5 min. Serum was prepared by allowing the blood to clot at room temperature for 50 min, then centrifuging at $1000 \times g$ for 10 min. Concentrations of endostatin and VEGF in the PRP, lysed PRP, PPP and serum were measured using specific ELISAs (ID Labs, London, Ontario, Canada). For measurement of VEGF and endostatin in serum from rats with ulcers in the stomach or colon, serum was prepared as described above.

Effects of platelets, plasma and serum on gastric ulcer healing

One group of rats was killed 3 days after ulcer induction to allow for determination of ulcer size at the time of initiation of drug treatment. Beginning on day 3 and continuing for 7 days, the rats were treated orally every 12 h with 0.5 ml of 0.9% saline, PRP, PPP, lysed PRP or serum. In some experiments, antibodies directed against VEGF or endostatin (both at 2.5 μg ml^{-1}) were added to the PRP or to saline before their administration to rats with ulcers. In other groups, rats with ulcers were treated with saline supplemented with VEGF (20 ng ml^{-1}), endostatin (80 mg ml^{-1}) or both. These concentrations of VEGF and endostatin represent the mean concentrations found in human PRP ($n = 5$).

Assessment of ulcer healing

On day 10 after ulcer induction, rats were euthanized by an overdose of sodium pentobarbital. The stomach was removed, opened by an incision along the greater curvature and photographed. The ulcer area was measured planimetrically (Ma *et al*., 2001a) in a blind manner.

Statistical analysis

All data are expressed as mean $\pm$ s.e.m., 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: TNBS from Fluka Chimika (Buchs, Switzerland); antibodies and ELISA kits for measurement of VEGF and endostatin from ID Labs (London, Ontario, Canada). All other supplies were from Fisher Scientific (Edmonton, Alberta, Canada).

Results

Induction of ulcers elicits a shift in plasma VEGF : endostatin to favour healing

Application of acetic acid to the serosal wall of the rat stomach resulted in the formation of an ulcer, which, by 3 days later, had a mean area of ~ 70 mm^2. This was accompanied by a shift in the relative levels of VEGF to endostatin in serum (Table 1), confirming previous findings (Ma *et al*., 2001a). Intracolonic administration of TNBS produced extensive colonic ulceration and inflammation, as described in detail previously (Morris *et al*., 1989; Wallace *et al*., 1989). As was the case for gastric ulceration, colonic damage was accompanied by decreased serum endostatin levels, resulting in an effective increase in the ratio of serum VEGF to endostatin (Table 1). An increase in serum VEGF : endostatin is consistent with promotion of angiogenesis and healing (Nagashima *et al*., 2000).

Table 1 Effects of gastrointestinal injury on serum levels of angiogenic factors

	Serum VEGF (ng ml^{-1})	*Serum endostatin* (ng ml^{-1})	*Ratio of VEGF : endostatin*
Before ulcer induction	18.2 $\pm$ 2.6	80.2 $\pm$ 3.5	0.23
3 days after ulcer induction	20.8 $\pm$ 1.3	56.6 $\pm$ 4.7*	0.37 (60% $\uparrow$)
Before colitis induction	18.5 $\pm$ 0.5	86.1 $\pm$ 8.2	0.21
3 days after colitis induction	16.3 $\pm$ 1.9	41.9 $\pm$ 13.3*	0.39 (86% $\uparrow$)

*$P < 0.05$ *versus* the corresponding 'before induction' group.

Intact platelets, not serum or platelet-free plasma, accelerate gastric ulcer healing

The gastric ulcers healed over the course of a week of twice-daily oral treatment with normal saline. The mean ulcer area was reduced by ~50% over this period. However, a significantly greater degree of ulcer healing occurred when rats were treated twice-daily with PRP (~80%; Figure 1). In contrast, rats treated orally with PPP or with serum for 1 week did not exhibit significant ulcer healing; that is, the area of ulcers was not significantly different from that observed before treatment. Administration of lysed PRP resulted in healing comparable to that in the group treated with saline.

VEGF is a potent angiogenic factor that has previously been suggested to play an important role in ulcer healing (Ma *et al*., 2001a, 2002; Deng *et al*., 2004). The ability of PRP (as opposed to PPP, serum or lysed PRP) to accelerate healing could not be attributed to differences in concentrations of VEGF in these preparations. As shown in Figure 2, the concentrations of VEGF did not differ among the PRP, serum and PPP. On the other hand, there were significantly higher levels of VEGF in the lysed PRP. Thus, there was no correlation between VEGF concentrations and effects of the different preparations on ulcer healing. It is possible, however, that the elevated concentrations of endostatin in lysed PRP, PPP and serum may have contributed to the reduced healing observed in rats treated with those preparations *versus* rats treated with PRP.

It is also noteworthy that PRP diluted by 50% with saline still exerted significant effects on ulcer healing (ulcer areas in diluted PRP group were 24.6 ± 2.6 *versus* $16.4 \pm 2.8\,mm^2$ in the undiluted PRP group and $35.3 \pm 2.8\,mm^2$ in the saline-treated group; both PRP-treated groups were significantly different from the saline-treated group, $P<0.05$, $n \geqslant 6$ in all groups). When PRP was diluted 10-fold with saline, a significant effect on ulcer healing, as compared to the saline-treated group, was no longer observed (mean ulcer area of $28.5 \pm 9.0\,mm^2$; $n=6$).

Physiological concentrations of VEGF and endostatin, given orally, do not affect gastric ulcer healing

Figure 3 summarizes further evidence to suggest that VEGF (or endostatin) within the PRP did not, by itself, account for the acceleration of gastric ulcer healing in rats treated with PRP. Treating rats with saline that had been supplemented with VEGF to match the concentration found in human PRP did not influence ulcer healing. Likewise, treating rats with endostatin-supplemented saline, or with saline supplemented with both VEGF and endostatin, did not influence gastric ulcer healing (Figure 3).

Platelet-mediated gastric ulcer healing is VEGF-dependent and endostatin-independent

To further examine the potential contributions of VEGF and endostatin to the effects of PRP on ulcer healing, studies were performed in which these growth factors were immunoneutralized within the PRP before its administration. As shown in Figure 3, immunoneutralization of VEGF reversed the beneficial effects of PRP on gastric ulcer healing. Indeed, the extent of healing in the group treated with PRP + anti-VEGF did not differ significantly from the healing observed at day 3 (before treatment). The anti-VEGF antibody, when given

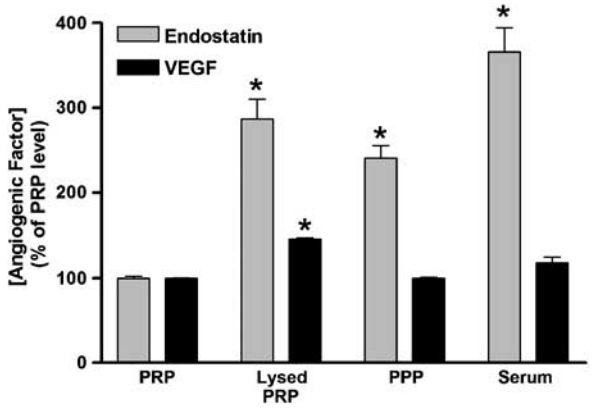

Figure 2 Serum and plasma levels of angiogenic factors. Concentrations of endostatin and VEGF in human PRP, lysed PRP, PPP and serum. The data are shown as a percentage of that in the PRP group ($20.2 \pm 1.8\,ng\,ml^{-1}$ of VEGF and $80.1 \pm 3.7\,ng\,ml^{-1}$ of endostatin). $^{*}P<0.05$ *versus* the PRP group. Each bar represents the mean ± s.e.m. for at least six samples.

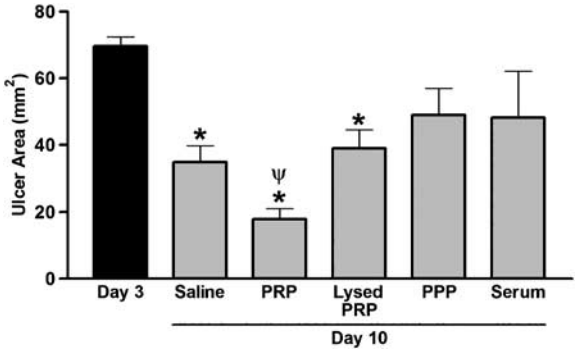

Figure 1 Acceleration of ulcer healing by PRP. Effects of twice-daily oral administration of 0.5 ml of human PRP, lysed PRP, PPP, serum or saline on gastric ulcer healing in rats. Treatments were initiated on day 3 and continued for 1 week. Gastric ulcer areas were measured on day 10. $^{*}P<0.05$ *versus* the day 3 (i.e., before treatment) group. $^{\Psi}P<0.05$ *versus* the saline-treated group. Each bar represents the mean ± s.e.m. for at least six rats.

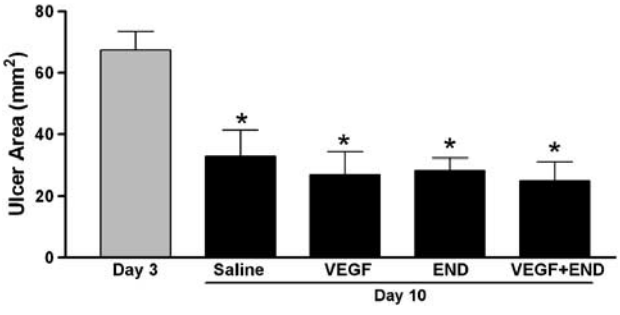

Figure 3 Exogenous VEGF and endostatin (END) do not accelerate ulcer healing. Lack of effect on gastric ulcer healing of saline supplemented with VEGF, END or both. The concentrations of VEGF and END matched those found in human PRP ($20\,ng\,ml^{-1}$ of VEGF, $80\,ng\,ml^{-1}$ of END). Groups of six rats were treated orally, twice-daily from day 3 to day 10 after ulcer induction, with 0.5 ml of saline or saline supplemented with VEGF and/or END. Gastric ulcer areas were measured on day 10. $^{*}P<0.05$ *versus* the day 3 group.

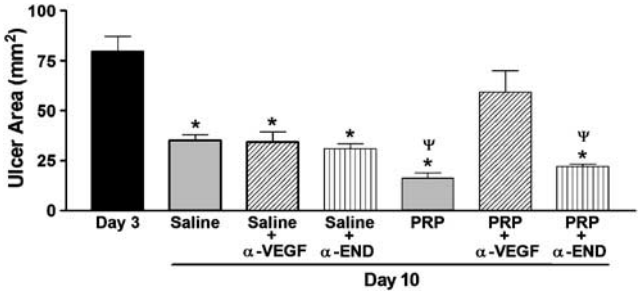

Figure 4 PRP accelerates ulcer healing *via* VEGF. Reversal of PRP-induced promotion of ulcer healing by immuno-neutralization of VEGF, but not endostatin (END). Groups of at least six rats were treated orally, twice-daily with 0.5 ml of saline or PRP. In some groups, anti-VEGF or anti-endostatin antibodies ($2.5\,\mu g\,ml^{-1}$) were added to the PRP or saline. Gastric ulcer areas were measured on day 10. $^{*}P<0.05$ *versus* the day 3 group. $^{\Psi}P<0.05$ *versus* the saline-treated group.

together with saline, did not affect ulcer healing relative to saline. Thus, the reversal of the effects of PRP on ulcer healing by anti-VEGF were attributable to immunoneutralization of VEGF within the PRP, rather than effects of the antibody at the site of ulceration. Pretreatment of PRP with anti-endostatin did not affect the pro-healing effects of PRP in the gastric ulcer model (Figure 4). Anti-endostatin also did not interfere affect gastric ulcer healing when administered in saline (Figure 4).

Discussion

Angiogenesis is an essential component of wound healing (Folkman *et al*., 1991; Schmassmann *et al*., 1995). Platelets are a rich source of both pro- and antiangiogenic factors, and are the first cell to accumulate at the site of injury. They are also the major source of VEGF in serum and may act as a transport system for VEGF (Verheul *et al*., 1997). VEGF induces angiogenesis by stimulating endothelial proliferation and migration. Previous studies from our laboratory have demonstrated the importance of platelets to gastric ulcer healing, and the importance of platelet and serum levels of VEGF (relative to endostatin) in that process (Ma *et al*., 2001a; 2002). In the present studies, we have extended these findings with the demonstration that orally administered human platelets can markedly accelerate gastric ulcer healing in rats. This effect was not observed with plasma depleted of platelets, or with serum. Immunoneutralization of VEGF in the PRP abolished its ability to promote ulcer healing. However, administration of exogenous VEGF, at a concentration matching that in human PRP, did not influence ulcer healing. Together with the observation that lysed PRP did not influence ulcer healing, this suggests that intact platelets are required in order for ulcer healing to be promoted, perhaps *via* their ability to 'present' VEGF to the site of injury.

Topical treatment with autologous PRP has been shown to be effective in several clinical situations (Carlson & Roach, 2002; Anitua *et al*., 2004; Weed *et al*., 2004). The present study is the first to report the beneficial effects of orally administered human PRP on experimental ulcer healing. Previous studies have shown that intravascular platelets contribute significantly to experimental ulcer healing, and that manipulation of their content of pro- *versus* antiangiogenic factors can influence healing (Ma *et al*., 2001a; 2002). The present study extended earlier observations (Ma *et al*., 2001a; 2002) that induction of an ulcer resulted, within 72 h, in increased platelet/serum ratio of VEGF to endostatin (i.e., to a more pro-angiogenic profile). Ulceration in the colon and stomach produced similar increases in this ratio, mainly owing to a decrease in serum endostatin. Shifts in the serum levels of pro- *versus* antiangiogenic factors have also been observed in arthritis, and have been suggested to be important in the repair of joint injury (Nagashima *et al*., 2000). The mechanisms responsible for ulcer-associated changes in serum/platelet levels of VEGF *versus* endostatin are not clear. Previous studies from our laboratory demonstrated that some drugs are capable of producing shifts in platelet/serum levels of VEGF *versus* endostatin in a direction opposite to that occurring following induction of an ulcer (i.e., decreased VEGF : endostatin). Our results suggested that these effects of drugs such as NSAIDs and ticlopidine contribute to their ability to impair ulcer healing (Ma *et al*., 2001a; 2002). These observations raise the possibility that pharmacological modulation of platelet growth factor content (or release) is a feasible approach to accelerate wound healing or to retard tumour growth.

Our finding that VEGF is crucial to the beneficial effects of platelets on ulcer healing is consistent with the finding that perforating gastrointestinal ulcers are a limitation to the use of anti-VEGF (bevacizumab) for the treatment of various types of cancer (Gordon & Cunningham, 2005). VEGF plays an important role in maintenance of gastrointestinal mucosal integrity. As in other tissues during ulceration (Arbiser *et al*., 2003; Pufe *et al*., 2003), VEGF and VEGF receptor expression is rapidly increased when ulceration occurs in the gastrointestinal tract (Akimoto *et al*., 2002; Baatar *et al*., 2002). Although we did not observe accelerated gastric ulcer healing when rats were treated with VEGF (or endostatin) at concentrations similar to those found in human PRP, others have shown that duodenal ulcer healing could be accelerated through intraduodenal or intravenous administration of adenoviral vectors or naked DNA transducing the VEGF gene (Deng *et al*., 2004). The reasons for the effectiveness of PRP in accelerating ulcer healing in a VEGF-dependent manner, *versus* the failure of physiological concentrations of VEGF to affect ulcer healing when administered in the same manner are not clear. It is possible that, after administration into the stomach, platelets released additional VEGF to what was measured in PRP. Second, it is possible that binding of the platelets within the ulcer bed was essential for local delivery of relatively high concentrations of VEGF. Third, it is possible that VEGF presented by the platelet in close proximity to the ulcer base may have been more protected from degradation by gastric acid than orally administered VEGF. The buffering capacity of plasma may have further protected VEGF from degradation. Also we cannot rule out a possible contribution of endostatin to the effects of various preparations of plasma/serum to ulcer healing. The high levels of endostatin in PPP and serum might have counteracted, to some extent, any beneficial effect that could be exerted by VEGF in those preparations. In previous studies of human umbilical vein endothelial cells, immunoneutralization of endostatin resulted in a marked increase in serum-stimulated angiogenesis and a marked decrease in serum-stimulated apoptosis (Ma *et al*., 2001a).

Reducing the number of platelets in the PRP preparation by half, through dilution, did not significantly diminish its ability to accelerate gastric ulcer healing. However, a reduction of platelet number in the PRP preparation to one-tenth resulted in the loss of the beneficial effects on gastric ulcer healing. Consistent with the notion that platelet number is another important determinant of their ability to influence healing processes, a single administration of thrombopoietin, on day 3 after ulcer induction, was found to result in a marked acceleration of gastric ulcer healing (Perini *et al*., 2005). It is possible, however, that this acceleration of healing may have been attributable to increased VEGF content within the platelet, as thrombopoietin is a potent stimulus for VEGF generation by the megakaryocyte (Mohle *et al*., 1997).

Platelets make an important contribution to ulcer healing, whether arriving at the site of injury *via* the vasculature or, as in the present study, delivered intraluminally. Clearly, this modulatory role of the platelet extends to other types of wounds, as well as to tumour growth. Although platelets do have some capacity to synthesize proteins, most of their content of growth factors is determined at the level of the megakaryocyte. Platelet content of pro- *versus* antiangiogenic factors can be manipulated pharmacologically (Mohle *et al*., 1997; Ma *et al*., 2001a; 2002). This raises the possibility that angiogenesis-dependent processes can be manipulated in a clinical setting through manipulation of platelet growth factor content and exploitation of the platelet as a targeted delivery system.

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