Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets

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The roles of proteinase-activated receptors (PARs) in platelet functions other than aggregation are not well understood. Among these is the release of factors that regulate the process of angiogenesis, such as endostatin and VEGF, which, respectively, inhibit and promote angiogenesis. PAR1 and PAR4 are expressed on the surface of human platelets and can be activated by thrombin. In the present study, we have attempted to determine the roles of PAR1 and PAR4 in regulating release of endostatin and VEGF from human platelets. Aggregation and endostatin release could be elicited by a specific PAR4 agonist (AYPGKF-NH_2). The PAR4 agonist concentration dependently suppressed VEGF release. A selective PAR1 agonist (TFLLR-NH_2) induced platelet aggregation and VEGF release but suppressed endostatin release. Thrombin did not affect endostatin or VEGF release. However, in the presence of a selective PAR1 antagonist (SCH79797), thrombin stimulated endostatin release and suppressed VEGF release. Conversely, in the presence of a selective PAR4 antagonist (transcinnamoyl-YPGKF-NH_2), thrombin stimulated VEGF release. *In vivo*, treatment of rats with established gastric ulcers with a PAR1 antagonist each day for 1 wk resulted in a significant retardation of healing. We conclude that PAR1 and PAR4 counter-regulate the release of endostatin and VEGF from platelets. These protease-activated receptors could therefore play a crucial role in regulating angiogenesis and in turn could regulate the processes of wound healing and tumor growth.

angiogenesis | aggregation | thrombin | protease

In addition to its central roles in blood coagulation and hemostasis, thrombin participates in a variety of biological processes, including inflammation and wound healing (1). Activation of platelets by thrombin is mediated at least in part through cleavage of proteinase-activated receptors (PARs). Four distinct PARs have been identified, with PAR1, PAR3, and PAR4 acting as receptors for thrombin. Human platelets express PAR1 and PAR4, and activation of either is sufficient to trigger platelet aggregation and secretion (2–5). A variety of bioactive substances, including growth factors and chemokines (6–8), are stored in platelets and released during activation. We have reported (9) that endostatin, a potent inhibitor of angiogenesis, is contained within rat platelets and released in response to thrombin via PAR4 in an aggregation-independent manner (10). In studies in rats, we demonstrated that pharmacological manipulation of platelet and/or serum levels of proangiogenic (VEGF) and antiangiogenic (endostatin) factors resulted in profound effects on healing of gastric ulcers (11, 12).

Whether human platelets contain endostatin is unknown. Moreover, the relative importance of PAR1 vs. PAR4 in regulating platelet endostatin release has not been reported. In the present study, we have demonstrated that human platelets contain endostatin, and that its release can be triggered by activation of PAR4 but not PAR1. Indeed, PAR1 activation leads to suppression of endostatin release but also to stimulation of the release of a proangiogenic substance, VEGF. PAR4 activation, in contrast, stimulates endostatin release and suppresses release of VEGF. Thus, PAR1 and PAR4 appear to act in a counter-regulatory manner to modulate release of factors regulating angiogenesis.

Methods

Preparation of Platelet-Rich Plasma (PRP). Human blood was taken from healthy volunteers with 3.4% sodium citrate (8:1 vol/vol). The volunteers denied ingesting aspirin or other nonsteroidal antiinflammatory drugs 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. The number of platelets in the PRP was counted by using a hemocytometer and adjusted to 2.5×10^8/ml with platelet-poor plasma.

PAR4 and Endostatin Release. Platelet aggregation and endostatin release were studied *in vitro* in response to various concentrations of AYPGK-NH_2 (AY-NH_2), a selective PAR4-activating peptide (PAR4-AP) (11, 13). Aliquots (0.4 ml) of the PRP were placed in the cuvette of a Chrono-Log (Havertown, PA) platelet aggregometer. The PRP was maintained at 37°C and was continuously stirred at 900 rpm. Three minutes later, AY-NH_2 (2–32 μM) was added to the platelet suspension in the absence or presence of transcinnamoyl (tcY)-YPGKF-NH_2 (tcY-NH_2; 400 μM), a PAR4 antagonist (10, 14), and aggregation was monitored for 5 min. The resulting platelet aggregate was centrifuged ($9{,}000 \times g$), and the supernatant was stored at −70°C until ELISA for endostatin were performed.

Regulation of Endostatin and VEGF Release by PAR1 and PAR4. Preliminary experiments were performed to determine the lowest concentrations of the PAR4-AP, thrombin, and TFLLR-NH_2, a PAR1-AP that would produce maximal aggregation of human platelets. Studies were then performed to identify concentrations of the PAR1-AP, PAR4-AP, and thrombin that elicited 25%, 50%, and 75% of maximal aggregation. Also, the highest concentration of the agonists that did not produce detectable aggregation was identified (referred to as 0% aggregation). Maximal aggregation was typically produced by concentrations of thrombin of ≈1 unit/ml, whereas concentrations of the PAR1-AP and PAR4-AP of 8 μM and 10 μM, respectively, were typically necessary to induce maximal aggregation.

Studies were then performed by using the three agonists at

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Abbreviations: PAR, proteinase-activated receptor; PRP, platelet-rich plasma; tcY, transcinnamoyl; PAR*n*-AP, PAR*n*-activating peptide.

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doses producing 0%, 25%, 50%, 75%, and 100% aggregation and at a concentration 50% greater than that identified as producing 100% aggregation (referred to as supramaximal). In parallel, the effects of each agonist concentration on release of VEGF and endostatin were determined.

Effects of PAR Antagonists on Thrombin-Induced Endostatin and VEGF Release. Suspensions of platelets were treated with a selective PAR1 antagonist (SCH79797; 3 μM) (15), a selective PAR4 antagonist (tcY-NH2, 400 μM) (10), or vehicle for 5 min before addition to the platelets of thrombin at a concentration producing maximal aggregation ($\approx$1 unit/ml). The effects of the pretreatments on thrombin-induced endostatin and VEGF release were then determined, as in the experiments described above. The concentrations of the two antagonists were selected on the basis of preliminary studies to determine the concentrations of each antagonist that selectively blocked the target PAR.

Effects of PAR Antagonists on PAR Agonist-Induced Endostatin and VEGF Release. Suspensions of platelets were treated with the selective PAR antagonists used in the experiments described above, or vehicle, for 10 min before addition to the platelets of a PAR1-AP (TFLLR-NH_2) or a PAR4-AP (AYPGK-NH_2), each at a concentration producing maximal aggregation ($\approx$8–10 μM). Endostatin and VEGF release were measured as described above.

Effects of a PAR1 Antagonist on Ulcer Healing. Gastric ulcers of a reproducible size were induced in rats by using the methods described in ref. 11. A subgroup of five rats was killed 3 days later for assessment of ulcer area. The remaining 12 rats were randomized to two groups and treated orally at 12-h intervals with SCH79797 (5 nmol in 0.5 ml of 0.9% saline) or vehicle (0.5 ml) for 7 days. On the final day, the rats were killed, and the ulcer area in each rat was determined in a blinded manner (11).

Statistical Analysis. All data are expressed as mean $\pm$ SEM, with sample sizes of four to five per group. Comparisons of data among groups were performed with ANOVA followed by the Student-Newman-Keuls test. An associated probability (*P* value) of $<$5% was considered significant.

Materials. Reagents were obtained from the following sources: AY-NH_2, tcY-NH_2, and TF-NH_2 were prepared ($>$95% purity) by solid-phase synthesis at the Peptide Synthesis Facility of the University of Calgary. Stock peptide solutions were prepared in 2.5 mM Hepes buffer, pH 7.4. Thrombin was obtained from Calbiochem, whereas SCH79797 [(*N*-3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7H-pyrrolo[3, 2-f]quinazoline-1,3-diamine)] was obtained from Tocris Cookson (Ellisville, MO). Thrombin was dissolved in sterile 0.9% saline, whereas SCH79797 was dissolved in 0.9% saline. Reagents for measurement of endostatin and VEGF were obtained from Chemicon.

Results

Exposure of human platelets to a PAR4 agonist (AY-NH_2) resulted in concentration-dependent aggregation and endostatin release (Fig. 1). Maximal endostatin release occurred at a concentration of the PAR4-AP that caused only $\approx$50% of maximal aggregation. Both aggregation and endostatin release were significantly attenuated by preexposure of the platelets to a PAR4 antagonist (tcY-NH_2), although at the higher concentrations of PAR4-AP, no effect of the PAR4 antagonist was evident.

Under basal conditions, human platelets released both VEGF and endostatin (Fig. 2). Stimulation with thrombin at concentrations of up to 50% greater than that necessary to induce maximal aggregation did not significantly affect endostatin or VEGF release. However, stimulation of platelets with a selective PAR1 agonist (TF-NH_2) resulted in a concentration-dependent

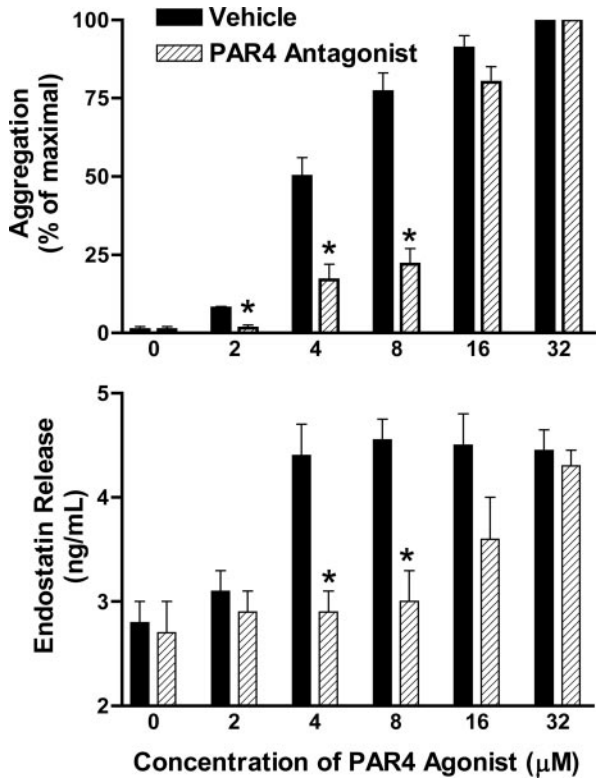

Fig. 1. Platelet aggregation (*Upper*) and endostatin release (*Lower*) induced by a PAR4 agonist (AYPGK-NH_2) and the effects of a selective PAR4 antagonist (tcYPGKF-NH_2; 400 μM). The platelets were exposed to vehicle (filled bars) or the PAR4 antagonist (hatched bars) for 5 min before exposure to the PAR4 agonist. *, $P < 0.05$ vs. the corresponding vehicle-treated group.

increase in VEGF release and decrease in endostatin release. In sharp contrast, a specific PAR4 agonist (AY-NH_2) caused the opposite effects, i.e., concentration-dependent increase in endostatin release and decrease in VEGF release. These results suggest that PAR1 and PAR4 on human platelets counter-regulate the release of VEGF and endostatin, and this result occurs independent of effects on aggregation. These observations also suggest that thrombin, at the concentrations tested, failed to significantly affect VEGF and endostatin release, because it activates both arms of this counter-regulatory system.

To further examine the hypothesis stated above, we examined the effects of selective antagonism of PAR1 and PAR4 on thrombin-induced VEGF and endostatin release. Although thrombin alone did not induce release of either growth factor, concurrent blockade of PAR1 with a receptor antagonist resulted in significant endostatin release and a significant reduction of VEGF release (Fig. 3). In contrast, stimulation with thrombin after exposure of the platelets to a PAR4 antagonist resulted in a significant increase in VEGF release above basal levels. Neither of the PAR antagonists alone affected basal release of VEGF or endostatin, but both attenuated thrombin-induced platelet aggregation (data not shown).

As shown in Fig. 4, the VEGF release that could be elicited by exposure of human platelets to a PAR1 agonist was abolished by previous exposure to a selective PAR1 antagonist, as was the suppression of endostatin release produced by the PAR1 agonist. However, the selective PAR4 antagonist did not significantly affect these responses to the PAR1 agonist. Conversely, the ability of a PAR4 agonist to suppress VEGF release and stimulate endostatin release was unaffected by preexposure of the platelets to a selective PAR1 antagonist but abolished by a

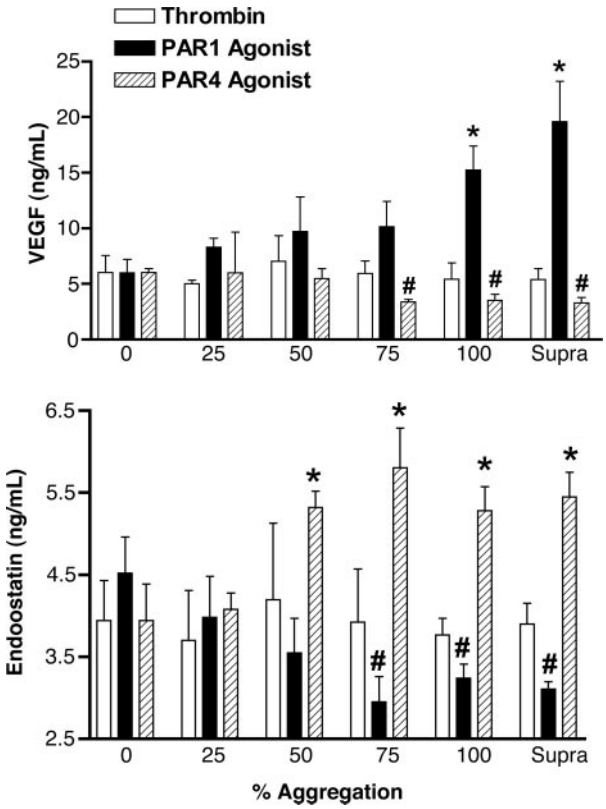

Fig. 2. Release of VEGF and endostatin from human platelets in response to thrombin or to specific PAR1 and PAR4 agonists (TFFLR-NH_2 and AYPGK-NH_2, respectively). Each agonist was tested at concentrations producing 25%, 50%, 75%, or 100% aggregation and at a concentration 50% greater than that causing maximal aggregation (Supra). *, Significant ($P < 0.05$) increase in release of the growth factor as compared with the group not treated with an agonist (0). #, Significant ($P < 0.05$) decrease in release of growth factor as compared with the group not treated with an agonist (0).

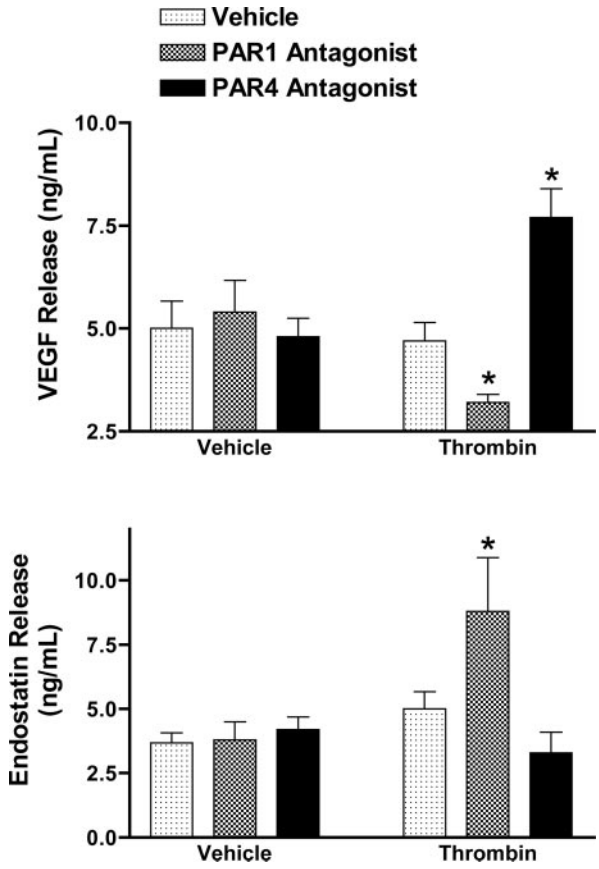

Fig. 3. Effects of selective PAR1 (SCH79797; 3 μM) and PAR4 (tcYPGKF-NH_2; 400 μM) antagonists on thrombin-induced release of VEGF and endostatin from human platelets. Platelets were incubated with one of the antagonists or vehicle for 5 min before stimulation with thrombin at the lowest concentration causing maximal aggregation (≈1 unit/ml). *, $P < 0.05$ vs. the corresponding vehicle-treated group.

selective PAR4 antagonist. These observations confirm the selectivity of these antagonists at the concentrations used. The concentration of SCH79797 used was shown in pilot studies to inhibit platelet aggregation and intracellular calcium signaling induced by TF-NH_2 (PAR1 agonist) but not to affect aggregation induced by AY-NH_2 (PAR4 agonist). The concentration of the PAR4 antagonist (tcY-NH_2) used in these studies significantly inhibited PAR4 agonist-induced aggregation but did not affect aggregation induced by the PAR1 agonist (TF-NH_2).

To determine whether PAR1 antagonism could influence the healing process *in vivo*, rats with established gastric ulcers were treated twice daily for 1 wk with a selective PAR1 antagonist (SCH79797) or vehicle. Rats treated with vehicle exhibited significant healing over the 1-wk treatment period (72% reduction of ulcer area) (Fig. 5). In contrast, significant healing of ulcers did not occur in the rats treated with the PAR1 antagonist.

Discussion

Angiogenesis is a crucial component of the wound-healing process and is also essential to tumor growth. The development of new blood vessels is driven by local release of proangiogenic factors such as VEGF, FGF, and EGF. However, angiogenesis can also be retarded or prevented by local release of antiangiogenic factors, one of the most potent of which is endostatin (16). Platelets can profoundly influence wound healing and tumor growth (17–19), at least in part through the release of pro- and antiangiogenic factors. We demonstrated in a rat gastric ulcer model that immunodepletion of platelets resulted in significant retardation of healing, whereas transfusion of platelets to thrombocytopenic rats restored normal healing (11). Moreover, we found that treatment with certain drugs (ticlopidine and celecoxib) caused in a shift in platelet and serum levels of endostatin vs. VEGF, with a corresponding shift in ulcer-healing rates (11, 12). Platelet release of VEGF and endostatin was responsible for the observed changes in endothelial proliferation and apoptosis (11).

Thrombin-induced endostatin release from rat platelets occurs via activation of PAR4 and independently of aggregation (11). In the present study, we have shown that, as in the rat, endostatin is released from human platelets after activation of PAR4. Furthermore, release of endostatin from human platelets is suppressed by activation of PAR1. At concentrations of up to 150% of those required for induction of maximal aggregation, thrombin did not elicit significant release of VEGF or endostatin. This observation is consistent with the fact that thrombin activates both PAR1 and PAR4 on human platelets (3). When PAR1 on platelets was blocked with an antagonist (SCH79797), thrombin stimulated endostatin release (a PAR4-like effect). When PAR4 on platelets was blocked with an antagonist (tcY-NH2), thrombin stimulated VEGF release (a PAR1-like effect). Thus, PAR1 and PAR4 act in a counter-regulatory manner to influence the release from platelets of two substances that can profoundly influence angiogenesis.

In a setting of injury or inflammation, exposure of platelets to proteases that activate PAR1 would preferentially release VEGF and suppress release of endostatin, thus favoring angiogenesis

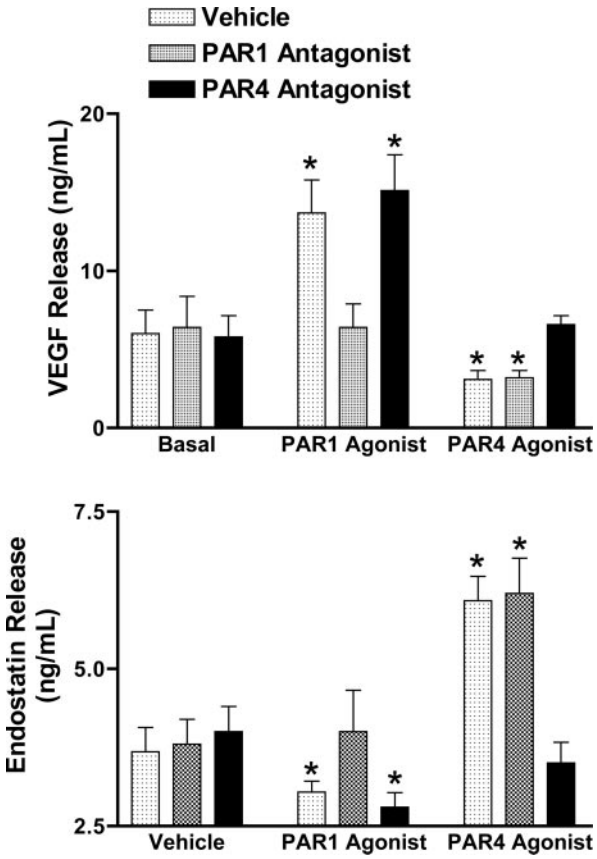

Fig. 4. Effects of selective PAR1 (SCH79797; 3 μM) and PAR4 (tcYPGKF-NH_2; 400 μM) antagonists on release of VEGF and endostatin from human platelets in response to selective PAR1 and PAR4 agonists (TFLLR-NH_2 and AYPGK-NH_2, respectively). Platelets were incubated with one of the antagonists or vehicle for 5 min before stimulation with the PAR1 or PAR4 agonist at the lowest concentration causing maximal aggregation (≈8–10 μM). *, $P < 0.05$ vs. the corresponding vehicle-treated group.

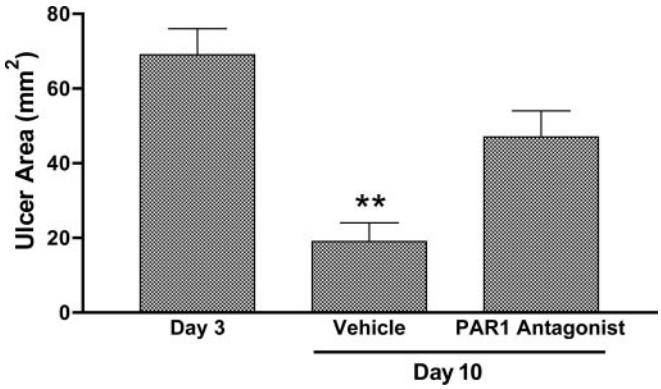

Fig. 5. Gastric ulcer area before (day 3) and after 1 wk of twice-daily oral treatment with a PAR1 antagonist (SCH79797; 5 nmol in 0.5 ml of 0.9% saline) or vehicle. Although significant healing was observed in vehicle-treated rats (**, $P < 0.01$ vs. the day 3 group), the mean ulcer area in rats treated with the PAR1 antagonist was not significantly different from that in the rats killed at day 3.

and wound healing. In contrast, exposure of platelets to proteases that activate PAR4 would preferentially release endostatin and suppress release of VEGF, thus impairing angiogenesis and wound healing. There is evidence to suggest that PAR1 activation does indeed lead to enhanced angiogenesis. For example, Yin *et al.* (20) demonstrated that PAR1 activation significantly enhanced angiogenesis and tumor growth in human melanoma cells. Moreover, PAR1 activation augmented expression of VEGF in melanoma cells, whereas a neutralizing Ab directed against VEGF blocked PAR1-induced proliferation of bovine aortic endothelial cells. In another study from the same group (21), PAR1 expression was found to correlate with invasion properties of breast carcinoma cells. Furthermore, human PAR1 antisense reduced the ability of cancer cells to migrate through an artificial matrix. In the present study, treatment of rats with a PAR1 antagonist was found to significantly impair the healing of gastric ulcers, consistent with a role for PAR1 activation in the process of wound healing. As mentioned above, previous studies from our laboratory have demonstrated that release of VEGF and endostatin from rat platelets can modulate angiogenesis and gastric ulcer healing (11). Rat platelets do not appear to express PAR1 (13) but do express a PAR1-like receptor that responds to PAR1 agonists (22). PAR1 is expressed by many cells other than platelets, including the vascular endothelium. We cannot rule out the possibility that actions of SCH79797 on ulcer healing could have been produced through effects on other cells expressing PAR1. For example, a recent study by Arisato *et al.* (23) demonstrated that release of VEGF from human smooth muscle cells could be stimulated by a PAR1 agonist. Of course, as with any pharmacological problem, it is possible that the effects on SCH79797 on ulcer healing occurred independently of interactions with PAR1.

Activation of either PAR1 or PAR4 is sufficient to trigger aggregation of human platelets (24), although they produce this effect through different mechanisms and with distinct kinetics (25). For example, PAR4-induced aggregation depends on adenosine diphosphate and is matric metalloproteinase 2-independent, whereas the reverse is the case for PAR1-induced aggregation. It is possible that PARs also regulate other platelet functions and may do so in a counter-regulatory manner, as observed in the present study.

Tumors can modulate PAR activity through the release of proteases that can either activate or disarm these receptors (26). Thus, tumors may modulate their own ability to grow by influencing the release of angiogenic factors from platelets and other cells. For example, tumors can release proteases, such as cathepsin (17), which are capable of activating or disarming PARs. The present studies raise the possibility that angiogenesis can be pharmacologically regulated through modulation of PARs expressed on targets such as the platelet. Thus, PARs may represent a viable therapeutic target for the regulation of wound healing and tumor growth.

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