Comparison of PAR-stimulated WPs.

HMEC-1 mediated by PAR-stimulated PRP releasates was delayed and significantly lower compared to the previously described differential release of VEGF and endostatin induced by the PAR-1- or PAR-4-stimulated platelets. Results: Activation of washed platelets (WPs) by PAR-1- or PAR-4-activating peptide (AP) promoted the VEGF and endostatin secretion in a concentration-dependent manner, being PAR-1-AP more potent than PAR-4-AP. The release of both molecules was abrogated by pre-incubation of platelets with PAR antagonists. Activation of platelet-rich plasma (PRP) with either PAR-1-AP or PAR-4-AP induced a significant VEGF secretion. Quantification of platelet-endostatin secretion was not possible in PRP due to the high levels of plasmatic endostatin vs. platelet content. Releasates from PAR-1- or PAR-4-activated WPs promoted similar pattern of angiogenic responses of HMEC-1 or EPC. Moreover, proliferation of platelet alpha-granules [3–5] and that both molecules can be differentially released upon platelet activation induced by the selective activation of protease-activated receptor (PAR)-1 or PAR-4 [3, 4, 6] providing a mechanism by which platelets could differentially modulate angiogenesis. Controversially, other studies provided evidences against this hypothesis [5, 7–11], suggesting that the release of VEGF and endostatin mediated by PAR-activation appears to be a stochastic but not a selective process that is regulated by the type and concentration of platelet agonist as well as the duration of platelet activation. We exhaustively characterized the VEGF and endostatin release from platelets activated with PAR-1-activating peptide (AP) and PAR-4-AP by comparing the same variables used in the controversial previous studies, including agonist concentrations, time of stimulation, washed platelets (WPs) or platelet-rich plasma (PRP) and the angiogenic activity of the PARs-stimulated platelet releasates.

Methods

Human platelets

Blood samples were obtained from healthy donors who had not taken non-steroidal anti-inflammatory drugs in the 10 days before sampling. This study followed principles in the Declaration of Helsinki and received the approval of the Institutional Ethics Committee and written consent from all the subjects. WPs (4 x 10^9/ml) and PRP were obtained as previously described [11] and then stimulated with human alpha-thrombin (Enzyme Research Laboratories, South Bend, IN), PAR-1-AP (TFLLR-NH2) or PAR-4-AP (AYPGKF-NH2) (Genbiotech SRL, Buenos Aires, Argentina).
Aires, Argentina). Platelet’s releasates were obtained by centrifugation of the stimulated platelets in the presence of Prostaglandin I_2 (PGI_2, 75 ng/ml) (Cayman, Ann Arbor, MI) and resting WPs were lysed as previously described [12]. The levels of VEGF and endostatin in platelet releasates were measured by ELISA (RayBiotech Inc., Norcross, GA).

**Angiogenic assays**

Human microvascular endothelial cells (HMEC-1) (ATCC Cell Lines) and human late outgrowth endothelial progenitor cells (EPC) were grown as previously described [11, 13]. Growth factor medium was replaced by supernatants of PAR-stimulated platelets and endothelial responses were assessed after 18 h. To evaluate the effect of plasma on cell growth and tubule formation, PRP (4 x 10^5 platelets/ml) was stimulated with PAR-1-AP or PAR-4-AP during 5 min, then centrifuged in the presence of PGI_2 and the calcium level was restored in the citrated plasma by the addition of CaCl_2 (22 mM). Endothelial proliferation was determined by measuring acid phosphatase activity. Wound healing of scraping monolayers of endothelial cells was analyzed with ImageJ software and the percentage of wound closure was calculated as [(wound area at 0 h - wound area at x h)/wound area at 0 h] x 100. Capillary tube formation in growth factor-reduced matrigel-coated plates (Becton Dickinson Biosciences, San Jose, CA) was examined under an inverted light microscope and the number of branch points was determined with ImageJ software (free from National Institutes of Health, Bethesda, MD).

**Statistics**

Results are expressed as mean ± SEM and were analyzed by one- and two-way analysis of variance followed by the Newman–Keuls multiple comparison test to determine significant differences between groups. A p value lower than 0.05 was considered to be statistically significant.

**Results**

**PAR-1 or PAR-4 activation induces the secretion of VEGF and endostatin from platelets**

Initially, we studied the release of VEGF and endostatin from WPs stimulated with each PAR-AP or thrombin. As shown in Figure 1(A), activation of platelets with each agonist induced the release of VEGF and endostatin in a concentration-dependent manner. Our results demonstrated that 10-times higher concentrations of PAR-4-AP than PAR-1-AP were required to induce similar levels of VEGF and endostatin release, indicating that PAR-1 activation exerts a more potent effect than PAR-4. The release of the angiogenic molecules was specifically mediated by PAR-1-AP and PAR-4-AP since pre-incubation of WPs for 30 min with SCH 79797 (10 μM) or tcY-NH_2 (400 μM), antagonists of PAR-1 and PAR-4, respectively, completely prevented the secretion of VEGF and endostatin (Supplemental figure). Low basal levels of both molecules were detected in releasates from unstimulated platelets, probably due to the washing procedure-induced activation (Supplemental figure). Since thrombin promotes the activation of PAR-1 and PAR-4 with different binding affinities [14, 15], we analyzed whether the differential release of VEGF and endostatin occurs in a thrombin-concentration manner. However, we found that both molecules were secreted at all thrombin concentrations assayed (Figure 1A).

To determine whether a differential release of VEGF and endostatin is occurring under a specific kinetic condition, we evaluated the secretion of both molecules using low and high concentrations of each PAR-AP or thrombin at different time points after platelet activation. As high concentrations of any stimuli induced maximal release of both molecules after 5 min, low concentrations required 60 min of platelet stimulation (Figure 1B–D). Interestingly, while the levels of VEGF and endostatin after PAR-1 and thrombin stimulation gradually increased over time (Figure 1B and D), the lower concentration of PAR-4-AP induced a low but significant release of both molecules during the first 30 min, reaching a peak at 60 min after platelet activation (Figure 1C). Overall, our results indicate that both molecules are unequivocally secreted from WPs upon PAR-1 or PAR-4 stimulation.

Because the differential release of VEGF and endostatin induced by PAR-activators has been observed in both WPs [3, 4] or PRP [6], we next evaluated the secretion of VEGF and endostatin in PRP. Figure 2 shows that similar to WPs, PRP activation by PAR-1-AP or PAR-4-AP resulted in the release of VEGF in a concentration-dependent manner, giving further support against a differential release of this molecule. However, when the secretion of platelet endostatin was analyzed, we found that the plasma levels of endostatin did not change after PRP stimulation. Interestingly and opposite to the very high ratio of platelet:plasma levels of VEGF (12 ± 1 vs. 1.3 ± 0.2 ng/ml, respectively), the total content of endostatin in platelets was 33-fold lower than in plasma (2.5 ± 0.3 vs. 83 ± 5 ng/ml, respectively) contributing negligibly to the presence of intra-platelet endostatin.

**Angiogenesis is promoted by PAR-stimulated platelet releasates**

Having demonstrated that PAR-1 or PAR-4 stimulation results in the release of pro- and anti-angiogenic molecules, we next evaluated the relevance of this phenomenon exploring the angiogenic potential of conditioned medium derived from WPs treated with each agonist. For this purpose, three sequential processes involved in vessel development were in vitro evaluated, including wound healing (Figure 3A), endothelial cell proliferation (Figure 3B) and reorganization into tubular structures (Figure 3C) [16] using the microvascular transfected cell line HMEC-1 and late outgrowth EPC. Our results demonstrated that all the angiogenic responses were similarly increased by supernatants from platelets stimulated with either PAR-1-AP or PAR-4-AP (Figure 3A–C). Similar results were observed for HMEC-1 and EPC indicating that different endothelial cell types are sensitive to the pro-angiogenic effect of PAR-stimulated platelets. The addition of non-stimulated platelet supernatants supplemented with each PAR-AP failed to trigger angiogenic responses (data not shown), indicating that the pro-angiogenic effects were not associated with a direct action of these peptides. These results demonstrate that even when the individual stimulation of PARs promotes the release of both pro- and anti-angiogenic factors, the overall effect is pro-angiogenic.

In order to understand the balance between pro- and antiangiogenic factors in a physiologic context, the effect of platelet poor plasma derived from resting or PAR-stimulated platelets in PRP on endothelial cell growth and tube formation was analyzed. Surprisingly and in contrast to the effect observed with releasates from PAR-stimulated WPs, proliferation and tube formation of HMEC-1 and EPC was not induced by PAR-stimulated PRP after 18 h (Figure 3D and E), suggesting that plasma either inhibit or delay the pro-angiogenic effect of platelet growth factors. In order to understand this intriguing result, angiogenic responses induced by supernatants from PAR-stimulated PRP or WPs were analyzed after 48 h incubation. Figure 3D and E shows that although proliferation and tubule formation of HMEC-1 and EPC were significantly increased after 48 h of incubation with releasates from stimulated PRP, both angiogenic responses were significantly lower than those
triggered by platelet releasates in the absence of plasma. Altogether, these results indicate that plasma exerts an anti-angiogenic modulation by delaying and interfering with the pro-angiogenic effect of platelet-derived growth factors.

**Discussion**

Currently, it is well established that platelets are involved in vessel development through the release of several angiogenic-factors from alpha-granules [1, 2]. In the context of the new theories regarding the release of these granules, and considering that platelets store both pro- and anti-angiogenic molecules, it has been postulated that these cells are able to induce selective functional angiogenic responses, through the specific release of VEGF and endostatin induced by the differential activation of PAR-1 or PAR-4 [3, 4, 6]. Controversially, other studies provided evidences against this hypothesis [5, 7–11].

![Figure 1](https://example.com/figure1.png)

**Figure 1.** PAR-1 and PAR-4 activations induce the secretion of intra-platelet VEGF and endostatin in a time- and concentration-dependent manner. (A) WPs (4 x 10^6/ml) were stimulated with the indicated concentrations of PAR-1-AP, PAR-4-AP or thrombin (Thr) for 5 min. Unstimulated WPs (−) were used as controls. (B) WPs were stimulated with indicated concentrations of PAR-1-AP (B), PAR-4-AP (C) or thrombin (Thr) (D) for 5, 30 or 60 min. Unstimulated WPs (0 min) were used as controls. VEGF (i) and endostatin (ii) levels in the supernatants were quantified by ELISA (n = 6, *p < 0.05, **p < 0.01, ***p < 0.001 vs. unstimulated. #p < 0.05, ##p < 0.01 vs. previous stimulation time).
Figure 2. Release of VEGF and endostatin induced by PAR-1-AP and PAR-4-AP in PRP. PRP (4 × 10⁹/ml) were stimulated with indicated concentrations of PAR-1-AP or PAR-4-AP for 5 min. Unstimulated PRP (−) was used as controls. VEGF (i) and endostatin (ii) levels in the supernatants of PRP, platelet poor plasma (PPP) and platelets lysates were quantified by ELISA (n = 6; *p < 0.05, ***p < 0.001 vs. unstimulated; ###p < 0.001 vs. lysis).

The different experimental conditions used in these studies hinder the understanding of the discrepancies obtained for each group. In this regard, some of these studies have been performed using WPs activated using either a unique stimulation time or concentration of PARs-AP [4, 5, 8–10, 17], impeding the full understanding of the dose-dependence or kinetic of the proposed differential release. Other works were performed using PRP [6, 7], adding a possible contribution of the molecules physiologically circulating in the plasma. In order to clarify these controversies, we exhaustively characterized the VEGF and endostatin release from platelets activated with PAR-1-AP and PAR-4-AP by comparing the same variables used in the previous studies and the overall angiogenic effect of PAR-stimulated platelets.

First, we studied by ELISA the ability of different concentrations of PAR-1-AP and PAR-4-AP to induce the release of VEGF and endostatin from WPs. In agreement with the established knowledge about the affinity of PARs with its respective activated peptide [14, 15], PAR-1-AP was powerful than PAR-4-AP to induce the secretion of platelet alpha-granules contain. In addition, the release induced by PAR-1-AP was faster than PAR-4-AP. Considering that granule secretion depends on intracellular calcium increases, the differences between PAR-1 and PAR-4 on granule secretion kinetic could be attributed to the well-described kinetic of intracellular calcium increase induced by these agonists, which consists in a rapid spike response induced by PAR-1, followed by a slower and prolonged response by PAR-4 [14]. Besides these differences, overall and in contrast to previous studies suggesting that VEGF and endostatin can be selectively released upon WPs PARs stimulation [3, 4], our data indicate that although the release of VEGF and endostatin varies according to the concentration of the stimuli and the time of platelet stimulation, both molecules are unequivocally secreted from WPs upon PAR-1 or PAR-4 stimulation. Similar to the WP experiments, the release of VEGF was also detected after the stimulation of PRP with either PAR-1 or PAR-4. In contrast, the secretion of endostatin was not observed after the stimulation of PRP by each PAR due to high levels of plasma endostatin compared with the intra-platelet amount of this molecule. These data suggest that even though a differential platelet endostatin release could occur using PRP, its contribution to the plasma level would be negligible, arguing again, against the possible selective release of platelet-derived endostatin induced by PAR-1 and PAR-4 activation. Considering that alpha-granules contain more than 300 proteins [18], a differential release induced by PAR stimulation could still be occurring with other molecules. However, two recent studies that analyzed 28 and 97 proteins by ELISA [8] or mass spectrometry [10], including VEGF and endostatin, showed that the most abundant alpha-granule proteins are similarly released after activation of each platelet PAR.

Finally, we demonstrated that even when the individual stimulation of PARs promotes the release of both pro- and anti-angiogenic factors, the overall effect is pro-angiogenic. In concordance with these data, we have previously described similar results using platelets stimulated by thrombin that induces platelet secretion through the combined action of PAR-1- and PAR-4-activation [11]. Noteworthy, no differences were observed between low and high concentrations of PAR-AP, suggesting that either the low amount of VEGF released is enough to induce a maximal angiogenic response or other angiogenic molecule(s) contribute to the observed effect. The latter hypothesis appears to be more suitable since we have recently demonstrated that angiogenesis mediated by platelets is mostly independent of VEGF and due to the combined action of several growth factors [11, 12]. In the same line of evidences, a recent study by Huang et al. showed that a complete rich angiogenic medium supplemented with releasates derived from either PAR-1- or PAR-4-stimulated platelets enhances pro-angiogenic activity of EPC due to a cooperation of multiple angiogenic regulators [17]. Interestingly, using a murine model they also demonstrated that albeit both PAR-stimulated platelets promoted the development of new vessels, PAR-1-derived supernatants were more potent that PAR-4 [17]. Whether this phenomenon occurs in humans remains to be investigated. Notably, we found that in contrast to the effect observed with releasates from PAR-stimulated WPs, endothelial angiogenic responses were delayed and inhibited by platelet poor plasma obtained after platelet stimulation with PAR-1 or PAR-4 in PRP. Having shown that plasma contains high levels of endostatin, which reversely interferes with the pro-angiogenic effect of several growth factors [19–21], it is conceivable that plasma endostatin, together with other plasmatic factors such as angiotatin, interleukin-10 and -12 [22], accounts for the lower angiogenic responses triggered by intra-platelet growth factors in PRP than in the absence of plasma.

In conclusion, our data show that VEGF is secreted after the stimulation of both, PAR-1 or PAR-4 in a concentration and time dependent-manner either using WPs or PRP. In contrast, the secretion of endostatin could not be determined using PRP and was only observed using WPs. This result was not associated with a differential alpha-granule release, but rather due to the high amount of endostatin in plasma that turns negligible the intra-platelet contribution. In spite that activation of platelets with either PAR-1-AP or PAR-4-AP promoted the release of pro- and anti-angiogenic molecules, the net biological effect was pro-angiogenic. Therefore, our results support the notion that while circulating endostatin accounts for the maintenance of a systemic
Figure 3. Releases from platelets activated with PAR-1-AP and PAR-4-AP trigger pro-angiogenic processes. (A–C) HMEC-1 or EPC (25 000 cells/well) were incubated during 18 h with platelets supernatants unstimulated (−) or activated by indicated concentrations of PAR-1-AP or PAR-4-AP during 5 min. (A) The wound healing of the scratched confluent HMEC-1 or EPC monolayers was analyzed under an inverted light microscope. (B) Endothelial proliferation was determined by measuring acid phosphatase activity after the addition of pNPP. (C) Tube formation in the matrigel-coated wells was analyzed under an inverted light microscope and the number of branch points was determined. Images are representative of four independent experiments. (D–E) HMEC-1 or EPC (25 000 cells/well) were incubated during 18 and 48 h with supernatants from unstimulated or PAR-stimulated PRP or WPs. (D) Endothelial proliferation was determined by measuring acid phosphatase activity after the addition of pNPP. (E) Tube formation in the matrigel-coated wells was analyzed under an inverted light microscope and the number of branch points was determined. (n = 4, **p < 0.01, ***p < 0.001 vs. unstimulated; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. PRP).
antiangiogenic state, locally, the release of platelet alpha-granule content promotes angiogenesis.

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Declaration of interest

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