Platelet-mediated angiogenesis is independent of VEGF and fully inhibited by aspirin

J Etulain[1]; C Fondevila[2]; S Negrotto[1]; M Schattner[1]

[1] Laboratory of Experimental Thrombosis, Institute of Experimental Medicine, CONICET-National Academy of Medicine. Buenos Aires, Argentina; [2] Haematology Service. Bazterrrica Clinic. Buenos Aires, Argentina.

S. N. and M. S. contribute equally to the study.

Corresponding Author: Mirta Schattner, PhD, Laboratory of Experimental Thrombosis, Institute of Experimental Medicine, CONICET-National Academy of Medicine, Pacheco de Melo 3081, 1425, Buenos Aires, Argentina

mschattner@hematologia.anm.edu.ar, mschattner@hotmail.com

Phone (+54-11)-4805-5759 ext 301

Fax (+54-11)-4805-0712

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SUMMARY

Background & Purpose: Platelets are major players in every step of vessel development through the local delivery of angiogenesis-modulating factors, including the proangiogenic vascular endothelial growth factor (VEGF) and the antiangiogenic endostatin. Although thrombin is the most potent agonist and is highly elevated in angiogenesis-related diseases, studies regarding its action on the release of platelet angiogenic factors are scarce and controversial. Herein, we have investigated the role of thrombin not only in VEGF and endostatin release but also in the net platelet angiogenic activity. **Experimental Approach:** Human platelets were stimulated with thrombin in the presence of inhibitors of signaling pathways involved in platelet activation and supernatants/releasates were used to determine the levels of angiogenic molecules and to induce angiogenic responses. **Key Results:** We found that thrombin induced the secretion of both VEGF and endostatin; however, the overall effect of the releasates was proangiogenic as they promoted tubule like formation and increased the endothelial proliferation. Both responses were only slightly suppressed in the presence of a VEGF receptor-neutralizing antibody. Pharmacological studies revealed that while inhibitors of PKC, p38, ERK1/2, Src kinases, or PI3K/Akt exerted only partial inhibitory effects, aspirin fully blocked the proangiogenic activity. **Conclusions & Implications:** In contrast to current belief, platelet proangiogenic responses are independent of VEGF and appear to be the result of the combined action of several molecules. Moreover, our data reinforce the notion that aspirin could be a promising therapeutic agent to treat angiogenesis-related diseases.

Keywords: Platelet, angiogenesis, VEGF, alpha-granules, Aspirin.

INTRODUCTION

Postnatal development of new blood vessels is mainly limited to sites of abnormal vascular surface. This process is regulated by a continuous interplay of stimulators and inhibitors of angiogenesis, and their imbalance contributes to numerous inflammatory, malignant, ischemic, and immune disorders (Carmeliet, 2005).

Platelets are being recognized as major players in every step of vessel formation (Patzelt *et al.*, 2012; Pipili-Synetos *et al.*, 1998) as they are a major storage of a broad array of growth factors, chemokines, cytokines, proteases, and cell adhesion molecules. Among the proangiogenic substances, platelets contain vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and stromal cell-derived factor-1 alpha (SDF-1α). Additionally, antiangiogenic molecules are secreted including thrombospondin-1 (TSP-1), endostatin, platelet factor-4 (PF-4), angiostatin, tissue inhibitor of metalloproteinases-1 and -4 (TIMP-1 and -4), and plasminogen activator inhibitor-1 (PAI-1) (Peterson *et al.*, 2010).

It has been recently demonstrated that angiogenic factors are packed into morphologically distinct populations of alpha-granules in megakaryocytes and platelets (Chatterjee *et al.*, 2011; Kamykowski *et al.*, 2011; Sehgal *et al.*, 2007; van Nispen tot Pannerden *et al.*, 2010) and can be differentially released based on selective engagement of platelet receptors, providing a mechanism by which platelets can locally and sequentially modulate angiogenesis (Bambace *et al.*, 2010; Battinelli *et al.*, 2011; Italiano *et al.*, 2008; Ma *et al.*, 2005). In this context, intraplatelet VEGF and endostatin have been the most studied as representative of pro and antiangiogenic factors, respectively, and they both can be differentially released upon platelet activation with different agonists such as ADP,

Thromboxane A_2, PAR-1 and -4 (Battinelli *et al.*, 2011; Italiano *et al.*, 2008; Ma *et al.*, 2005). Surprisingly, although thrombin is the most potent known physiologic agonist and is highly elevated in several pathological conditions where angiogenesis occurs (e.g., cancer and inflammation) (Han *et al.*, 2011; Martorell *et al.*, 2008), its action on the release of angiogenesis-modulating factors from platelet granules is still a matter of controversy.

In the present study, we aimed to further investigate the release of VEGF and endostatin mediated by thrombin, elucidate the role of VEGF in the overall angiogenic effect of releasates derived from thrombin-activated platelets as well as the effect of pharmacological inhibition of the main signaling pathways involved in platelet activation. Our results show that supernatants derived from thrombin-stimulated platelets efficiently **trigger** both VEGF and endostatin, but the net biological activity of the releasates was proangiogenic as measured by induced endothelial cell proliferation and capillary tube formation. These angiogenic responses were independent of the action of VEGF and were mainly due to the combined action of several intraplatelet proangiogenic molecules. Moreover, our data showing that the proangiogenic activity of platelets was fully blocked by aspirin (ASA) strengthen the notion that this drug could offer promising, therapeutic relief for angiogenic-related diseases.

METHODS

Preparation of 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 received the approval of the

Institutional Ethics Committee and written consent from all the subjects. Platelet rich plasma (PRP) was obtained from anticoagulated blood (sodium citrate 3.8%) by centrifugation at 180 x *g* for 10 min. Platelets were washed as described previously (Etulain *et al.*, 2011), PRP was centrifuged in the presence of PGI_2 (75 nM) at 890 x *g* for 10 min, washed in washing buffer (pH 6.5) at 890 x *g* for 10 min, and resuspended in Tyrode's buffer at a concentration of 4 $x10^8$ ml^{-1}. $CaCl_2$ (1 mM) was added 1 min before platelet stimulation.

Experimental design

Washed platelets (WPs) were stimulated with human alpha-thrombin (Enzyme research laboratories, Swansea, UK) for 5 min. Then, cells were centrifuged twice (first at 1100 x *g* for 5 min and then at 9300 x *g* for 5 min) in the presence of PGI_2 (75 nM), and supernatants stored at -80ºC until assayed. In selected experiments, WPs were incubated for 30 min with selective inhibitors of cyclooxygenase (ASA) (Sigma, San Diego, CA, USA), PKC (Gö-6983), p38 (SB-203580), ERK1/2 (U-0126), Src kinases (PP1), or PI-3K/Akt (LY-294002) (Enzo Life Sciences International, Inc. San Diego, CA, USA). The drug and molecular target nomenclature conform to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011). The concentrations of the p38, ERK, Src, Akt, and PKC inhibitors were selected from pilot studies and were the minimal that completely suppressed phosphorylation of the specific target proteins (Supplemental Figure 1). The concentration of ASA or indomethacin used was the minimal that abrogated arachidonic acid but not thrombin-induced platelet aggregation (data not shown).

Immunoblotting

WPs (1 x10^8 ml^{-1}) were lysed in loading buffer in the presence of a protease inhibitor cocktail (Sigma). Equivalent amounts of proteins were subjected to electrophoresis on a 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking, the membranes were incubated overnight at 4 ºC with primary antibodies (pSrc-Tyr416, pp38-Thr180/Tyr182, and Phospho-(Ser) PKC Substrate were from Cell Signaling, Danvers, MA, USA; pERK E-4 and pAkt1/2/3-Ser473 were from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by an HRP-linked secondary antibody (Santa Cruz Biotechnology) for 1 h at 22ºC. Protein bands were visualized by using the ECL reaction. Immunoblotting results were semi-quantitated using GEL-PRO ANALYZER 3.1 software, Media Cybernetics, Inc. Bethesda, MD, USA and values from blot reprobes were used for normalization of data for protein loads.

Measurement of angiogenic factors by ELISA or protein array

The levels of angiogenic factors in platelet releasates were measured by using ELISA commercial kits (VEGF and endostatin) or a human angiogenesis antibody array (G series 1000 from RayBiotech, Inc. Norcross, GA, USA), according to the manufacturer's instructions.

Platelet ATP release

ATP levels were measured in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA) under stirring conditions as previously described (Etulain *et al.*, 2012). ATP was also

measured under non-stirring conditions and its levels were calculated at the end of the assay by adding a known amount of ATP (Sigma, San Diego, CA, USA).

Measurement of Thromboxane B_2 (TXB_2) release

WPs were incubated with thrombin for 5 min in an aggregometer with or without stirring at 1000 rpm. Addition of ice-cold PBS containing EDTA (2 mM) and ASA (500 μM) was used to stop the reaction. The samples were centrifuged and TXB_2 was measured in the supernatants using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA) (Etulain *et al.*, 2011).

Determination of P-selectin expression

WPs were incubated with or without inhibitors, stimulated, fixed with paraformaldehyde (1%), and stained with a FITC-CD62P (anti-P-selectin) (BD Biosciences, San José, CA, USA) in phosphate buffered saline (PBS) 0.1% FBS solution or an equivalent amount of isotype matched control antibody. Samples were analyzed by flow cytometry on a FACSCalibur flow cytometer® and using CELLQUEST software (BD Biosciences, FranklinLakes, NJ, USA) (Etulain *et al.*, 2012).

Endothelial cell culture

Human microvascular endothelial cells (HMEC-1) were obtained from the Center of Disease Control and Prevention (CDC, Atlanta, USA). Cells were grown in RPMI supplemented with fetal bovine serum (10 %), L-glutamine (2 mM) and streptomycin (100 μg ml^{-1}), and penicillin (100 U ml^{-1}) at 37ºC in a humidified 5% CO_2 incubator. In selected

experiments the VEGF receptor (VEGFR) was blocked with a monoclonal neutralizing antibody against the VEGFR-2 (R&D Systems, Minneapolis, MN, USA). Excess antibody was removed by exhaustive washing before the platelet supernatants were added to the endothelial cultures.

Endothelial viability assay

Cells were analyzed for changes in morphology and viability by labeling cells with a mixture of the fluorescent DNA binding dyes (acridine orange and ethidium bromide) as previously described (Negrotto *et al.*, 2006).

Endothelial cell proliferation

Proliferation was determined by MTT [3-(4,5-dimethylthiaxol-2-yl)-2,5-diphaenyltetrazolium bromide] assay (Promega Corporation, Madison, WI, USA). HMEC-1 cells were synchronized in Tyrode's buffer with FBS (1 %) overnight, washed and incubated with platelet supernatants for 18 h before the addition of MTT solution for 3 h at 37ºC. Absorbance was measured at 570 nm and the number of cells was extrapolated from a standard curve performed for each experiment. The incubation time was determined in initial experiments and represents the population doubling time of HMEC-1 after the addition of thrombin-stimulated platelet supernatants. Positive and negative controls were Tyrode's buffer with and without recombinant VEGF (20 ng ml^{-1}, R&D Systems), respectively.

Capillary tube formation assay

HMEC-1 were seeded in growth factor-reduced matrigel-coated plates (Becton Dickinson Biosciences, Bedford, MA, USA) and exposed to platelet supernatants for 18 h. Tubule formation was examined under an inverted light microscope and the number of branch points in four non-overlapping fields was measured. Images were taken in a Nikon microscope equipped with a Nikon 100X/1.4 NA objective and a 100-W mercury lamp. Images were acquired with a Nikon camera and analyzed with Image J software (Arnaoutova *et al.*, 2009).

Statistical analysis

The results are expressed as the mean ± SEM and were analyzed by one-way analysis of variance followed by 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

Thrombin induces the secretion of intraplatelet VEGF and endostatin

To clarify whether thrombin induces a differential release of VEGF and endostatin, we first measured the levels of both molecules in supernatants derived from platelets activated by different concentrations of thrombin. Figure 1 shows that thrombin was able to induce the secretion of both VEGF and endostatin in a concentration-dependent manner with a similar EC_{50} for both proteins (0.051±0.005 and 0.05±0.01, respectively). To evaluate whether the release of both molecules was dependent on the aggregation process, the experiments were repeated under non-stirring conditions. Figure 1A shows that **the release of VEGF under stirring conditions was significantly higher than non-stirring conditions only at low concentrations of thrombin. Regarding endostatin, no differences were observed between both conditions.** It has previously been reported that activation of human platelets with ADP stimulates the release of VEGF but not endostatin, whereas TXA_2 triggers the release of endostatin but not VEGF (Battinelli *et al.*, 2011). Because thrombin-induced platelet activation results in ADP release and TXA_2 generation, to further understand the interplay of both agonists on the release of platelet-derived angiogenic molecules, we next inferred the levels of ADP (by measuring ATP), and TXA_2 (by measuring its stable metabolite TXB_2) in the supernatants of platelets stimulated with the EC_{50} of thrombin capable of inducing the release of VEGF and endostatin. The secretion of VEGF and endostatin mediated by thrombin, from stirred or non-stirred platelets, was accompanied by ATP release (Figure 1B) and TXB_2 formation (Figure 1C).

Releasate from platelets activated with thrombin has an overall proangiogenic effect that is mostly a VEGF-independent response

After demonstrating that thrombin-stimulated platelets secreted both VEGF and endostatin, two factors that exert opposing effects on vessel formation, we next studied the overall angiogenic potential of platelet releasates after thrombin stimulation, and endothelial biological responses such as proliferation and capillary-like tubule formation. We found that as with VEGF, the releasates derived from platelets stimulated with low and high thrombin concentrations significantly increased both angiogenic responses (Figure 2A and B). In contrast, supernatants derived from resting platelets or thrombin without platelets showed no effect. To determine the contribution of VEGF to the overall proangiogenic effect of platelet releasates, we repeated these experiments in the presence of a VEGF receptor-neutralizing antibody. Surprisingly, under this condition, both endothelial proliferation and tubule formation induced by releasates from thrombin-activated platelets were only slightly suppressed (13-17% of inhibition vs. control, Figure 2C and D). Notably, this antibody exerted a full inhibitory effect (96-98%) on angiogenic responses that were induced by recombinant VEGF (20 ng ml^{-1}) (Figure 2C and D). These findings indicate that although intraplatelet VEGF contributes to the overall proangiogenic effect triggered by thrombin-activated platelet releasates, it is not essential.

Levels of angiogenic proteins in releasates of thrombin-activated platelets

Based on our findings showing that the positive regulation of angiogenesis mediated by platelet releasates is a VEGF-independent action, we next aimed to identify other angiogenic factors responsible for the observed proangiogenic effect. Therefore, the levels

of angiogenic proteins in supernatants derived from thrombin-activated platelets were compared to those from resting platelets using a human angiogenesis antibody array (absolute mean of fluorescence intensity values in Supplemental Table). An analysis of the array data showed that similar to the results obtained by ELISA, VEGF and endostatin secretion was augmented in supernatants from platelets stimulated by thrombin (Supplemental Table) compared to supernatants from unstimulated platelets. Additionally, some of the most elevated proangiogenic proteins included IL-6 and TPO (500-1000-fold increase) as well as angiopoietin-1 and -2, G-CSF, IL-2, GM-CSF, GRO, IL-1 alpha and beta, TNF-alpha, MCP-3 and -4, I-309, IL-8, Tie-2, MMP-1 and -9, and ENA-78 (5-40-fold increase). Factors such as IGF-1, MCP-1 and EGF were moderately augmented (2-5-fold increase) whereas the levels of angiogenin, PDGF-BB and RANTES were similar to unstimulated controls (Figure 3).

Signaling pathways involved in angiogenesis-mediated by platelets

To explore the intracellular signals involved in the secretion of angiogenic molecules from thrombin-stimulated platelets, we next tested specific inhibitors of the major signaling pathways involved in platelet granule secretion including the cyclooxygenase-1 (ASA), PKC (Gö-6983), p38 (SB-203580), ERK1/2 (U-0126), Src (PP1), and PI3K/Akt (LY-294002) pathways (Li *et al.*, 2010). Immunoblot studies show that all the inhibitors at the concentration employed completely blocked activation of their respective targets (Supplemental Figure 1). As shown in Figure 4, interference with each of the main signaling pathways resulted in different degrees of inhibition of the angiogenic responses induced by platelet supernatants. For instance, endothelial cell growth and tubule formation

were partially, but significantly inhibited by the blockade of PKC, p38, and ERK1/2 whereas inhibition of Src kinases or PI3K/Akt showed little or no effect, respectively (Figure 4A and B). However when platelets were activated in the presence of ASA, both processes were completely suppressed (96% inhibition). Because ASA can exert antiangiogenic effects in a COX-independent manner (Borthwick *et al.*, 2006) we also analyzed the effect of indomethacin, a non-salicylate alternative inhibitor of COX. Like ASA, incubation of platelets with indomethacin resulted in a complete inhibition of the platelet proangiogenic activity (Figure 4D and E). Moreover, treatment of platelets with ASA did not significantly inhibit the phosphorylation of p38, ERK, Src, Akt, and PKC substrates (Supplemental Figure 1).

Although it could be argued that the inhibition of angiogenesis was a direct action of these different compounds on the endothelial cells, this hypothesis was ruled out because the addition of thrombin-stimulated platelet supernatants, which were supplemented with the inhibitors after platelet stimulation to endothelial cultures, failed to modify any of these angiogenic responses. Higher concentrations were required to inhibit these processes (Supplemental Figure 2A and B). Moreover, taking into account that ASA was the most powerful inhibitor and is an irreversible inhibitor of COX, we performed additional experiments using supernatants from platelets that were preincubated with ASA, washed, and then stimulated with thrombin. As shown in Supplemental Figure 2C and D, platelet-mediated angiogenesis processes were completely inhibited by ASA even when the drug was removed before platelet activation.

Because signaling pathways involved in secretion may depend on the strength of stimulus, we next analyzed whether a similar effect was observed using a higher thrombin

concentration. Although the drug efficiency in angiogenesis inhibition triggered by thrombin 1U ml^{-1} was lower than the observed effect with thrombin 0.05 U ml^{-1}, the inhibition pattern induced by each drug was similar for both agonist concentrations, and ASA remained the most effective blocker of platelet-mediated angiogenesis (Figure 4A and B).

Furthermore, the observation that necrotic or apoptotic cells never exceeded 1% for all treatments indicated that the inhibition of cell growth was not associated with a drug mediated cytotoxic effect **(Supplemental Figure 3).**

Altogether, these data demonstrate that the net proangiogenic effect induced by platelet-derived soluble angiogenesis modulators is regulated by distinct signaling pathways and largely depends on cyclooxygenase-1 action.

Alpha granules are not all identical; they have different protein content and their release is regulated by different signaling pathways.

As it has been demonstrated that not all alpha granules have the same protein content and that their release can be regulated by different signaling pathways, we aimed to identify which molecules within the platelet granule cargo were affected by each specific inhibitor. To this end, the secretion pattern of platelet angiogenesis-modulating substances triggered by thrombin was analyzed before and after the blockade of the main signaling pathways. Consistent with the results observed in the endothelial responses, incubation of platelets with the different inhibitors resulted in a selective blockade of the secretion of angiogenic molecules (Supplemental Table). Among the 26 different proangiogenic molecules detected by the array, the degree to which IL-1-beta and -2, Angiopoietin-1 and -2, G- and GM-CSF,

and TNF-alpha were inhibited most closely followed the inhibition pattern observed in the biological endothelial responses (Figure 5A), suggesting that these molecules are crucial mediators of the proangiogenic effect exerted by releasates from thrombin-activated platelets. Nevertheless, considering that ASA was the most effective inhibitor of the biological responses (Figure 4), we also analyzed the array data to determine whether there was any molecule inhibited only by ASA and found that the release of ENA-78 and EGF were solely but moderately (20 and 22% of inhibition, respectively) blocked by the inhibition of cyclooxigenase-1 enzyme (Supplemental Table).

Interestingly, the array data showed that the inhibitors of PKC, p38, ERK1/2, Src and PI3K/Akt partially inhibited VEGF secretion (between 20 and 50% inhibition) by thrombin-activated platelets, whereas ASA had almost no effect (Figure 5B). These findings were confirmed by ELISA studies (Figure 5B).

To analyze whether this differential signaling regulation also influences other non-angiogenic molecules stored either in alpha or dense granules, the exposure of P-selectin and the ATP release induced by thrombin was evaluated in the presence of the different inhibitors. Figure 5C shows that as with VEGF release, inhibition of PKC activation resulted in a 50% reduction of both the exposure of P-selectin and ATP secretion. However and in contrast to alpha granule release (either VEGF or P-selectin), the blockade of Src and PI3K/Akt pathways had the greatest impact on ATP release, as it was reduced by more than 50% of control values (Figure 5D).

DISCUSSION AND CONCLUSIONS

Platelets, in addition to maintaining haemostasis, play a critical role in regulating angiogenesis by releasing factors that promote the growth of new vessels. Although thrombin is the most powerful platelet agonist and is generated in almost all physiopathological processes involving the formation of new vessels (Moser, 2008), the thrombin-dependent release of pro- and antiangiogenic molecules is not clearly understood. Pioneering studies by Mohle (Mohle *et al.*, 1997) and Maloney *et al* (Maloney *et al.*, 1998) demonstrated that megakaryocytes and platelets synthesize and release VEGF upon stimulation with thrombin. However, a subsequent study showed that platelet activation with thrombin triggers the secretion of neither VEGF nor endostatin because it simultaneously activates protease activated receptors (PAR)-1 and -4, which counter-regulate the release of both molecules (Ma *et al.*, 2005). In this study, we have investigated the role of thrombin in the differential release of platelet VEGF and endostatin as well as the net platelet angiogenic activity. We found that thrombin triggered the secretion of both angiogenic molecules in a concentration-dependent manner, whereas platelet releasates promoted endothelial cell proliferation and capillary-tube-like structures in a VEGF-independent but TXA_2-dependent manner. While Maloney *et al* (Maloney *et al.*, 1998) described that the release of VEGF was intimately associated with platelet aggregation, our present results performed under stirring or non-stirring conditions show that secretion of either VEGF or endostatin can occurs independently of the platelet aggregation response. **Although stirring increased VEGF release it did not modify the secretion of endostatin**. Similar results concerning the release of VEGF were observed by Mohle *et al* (Mohle *et al.*, 1997). The differential release of VEGF and endostatin was observed not only after PAR-1 and PAR-4 activation (Italiano *et al.*, 2008; Ma *et al.*, 2005) but also upon

ADP or TXA_2 platelet activation (Bambace *et al.*, 2010; Battinelli *et al.*, 2011). These findings raise the following question: what would be the net biological response when platelets are simultaneously stimulated with ADP and TXA_2? Our present data show that when both mediators are generated as a result of platelet stimulation with thrombin, both VEGF and endostatin are secreted and that the net biological activity (evaluated as endothelial proliferation and capillary-tube-like structures) is distinctly proangiogenic.

Surprisingly and contrary to current belief, we also found that the role of VEGF appears to be negligible in the platelet-mediated angiogenic activity. A blockade of the endothelial VEGF receptor had only a weak inhibitory effect (15% inhibition) on the entire angiogenic response mediated by releasates from thrombin-stimulated platelets. It is widely recognized that in addition to VEGF and endostatin, platelet alpha granules contain several growth factors and chemokines that positively and negatively regulate angiogenesis (Peterson *et al.*, 2010). We demonstrated that after thrombin stimulation, the proangiogenic proteins that are elevated the most included not only VEGF but also IL-6 and TPO (500-1000-fold increase) as well as angiopoietin-1 and -2, G-CSF, IL-2, GM-CSF, GRO, IL-1 alpha and beta, TNF-alpha, MCP-3 and -4, I-309, IL-8, Tie-2, MMP-1 and -9, and ENA-78 (5-40-fold increase).

Thus, our data indicate that the net proangiogenic activity is the result of the combined action of several cytokines, which was further confirmed by our pharmacological studies to elucidate the molecular signaling involved in the release of these angiogenic molecules. Proliferation of endothelial cells and the formation of new vessels were completely suppressed by platelets treated with ASA prior to thrombin stimulation, were partially blocked by the inhibition of the PKC, p38, and ERK pathways, and were barely affected in

platelets pre-incubated with drugs against Src kinases and the PI3/AKT pathway. The observation that the inhibition patterns induced by each drug were similar (although less efficient in the degree of effect) when platelets were stimulated with either 0.05 or 1 U ml^{-1} thrombin concentration, indicates that regulation of platelet-mediated angiogenesis by this signaling pathway is not significantly modified by the strength of stimulus. These results are in agreement with those of Coppinger et al who demonstrated that the releasate profile observed when platelets were stimulated with high concentrations of ADP was identical to that seen with lower concentrations, although the effect of ASA was more pronounced in platelets stimulated with the lower dose of ADP (Coppinger *et al.*, 2007). Furthermore, an analysis of the individual angiogenic molecules revealed that the inhibition of IL-1-beta and -2, Angiopoietin-1 and -2, G- and GM-CSF and TNF-alpha release by the different compounds correlated with a similar inhibition pattern observed in the proliferation of endothelial cells and tubule-like formation. Nevertheless, considering that ASA was the most effective inhibitor of the biological responses (>95% Figure 4) it is conceivable that other molecules not represented on the array-based screening experiments might be involved.

The intriguing observation that the inhibition of specific signaling pathways of platelet activation results in a differential proangiogenic molecule release could be explained by the new theories of platelet granule secretion. While some recognize the existence of alpha granule subtypes with different morphology and protein cargo that can be differentially released in response to a specific agonist (Chatterjee *et al.*, 2011; Italiano *et al.*, 2008; van Nispen tot Pannerden *et al.*, 2010), recent studies show that alpha granule content proteins are stochastically packaged into subdomains within single granules, and that proteins

displayed little, if any, pattern of functional co-clustering (Kamykowski *et al.*, 2011). Platelet secretion, rather than having a limited thematic response to specific agonists, appears to be a stochastic process potentially controlled by several factors, such as cargo solubility, granule shape, and/or granule-plasma membrane fusion routes (Jonnalagadda *et al.*, 2012). Although the structure and dynamics of alpha granule secretion appear somewhat controversial and still remains to be clarified, the different theories seem to converge in the notion that selective signaling pathways might be involved in the regulation of alpha granule content exocytosis.

Our finding showing that cell proliferation and tubule formation are similarly affected by the different inhibitors could be somewhat surprising considering that the process of tubule formation on a basement membrane matrix is mainly supported by migration and differentiation, but not by proliferation of endothelial cells. Nevertheless, like our, several studies have shown a similar inhibition pattern of tubule formation and proliferation mediated by several pharmacological inhibitors, suggesting that the intracellular pathways governing the tubule formation and proliferation are not so distinctly separated and some may even overlap (Kim *et al.*, 2008; Wang *et al.*, 2005; Yu *et al.*, 2006). Still, we cannot rule out the possibility that the unifying elements driving the effect of our compounds on the endothelial cell responses are lipids products of COX, even TXA_2, or platelet microparticles rather than a specific alpha granule derived protein.

Regarding VEGF, although numerous studies have suggested that this molecule plays a major role in platelet-mediated angiogenesis, we here show that the selective blockade of this growth factor had almost no effect. Moreover, our data showed that ASA failed to inhibit VEGF release but fully blocked endothelial proliferation and tubule formation.

Overall, our study introduces the novel concept that VEGF is not essential for the proangiogenic activity of platelets, and reinforces the notion that there are multiple, redundant pathways mediating this process.

Although angiogenesis is a crucial component of the wound healing process, it also occurs in several diseases including cancer. The tumor milieu is enriched in angiogenic factors derived from tumor, endothelial and stromal cells, or by recruited myeloid populations of tumor-associated macrophages or neutrophils (Weis *et al.*, 2011). However, several *in vitro* and *in vivo* studies in animals and patients have highlighted that platelets are other relevant source of angiogenic factors that contribute to tumor progression (Cho *et al.*, 2012; Ho-Tin-Noe *et al.*, 2008; Klement *et al.*, 2009; Peterson *et al.*, 2012; Pietramaggiori *et al.*, 2008; Sabrkhany *et al.*, 2011). Therefore, pharmacological inhibition of platelet proangiogenic activity has been considered as a potential adjuvant therapy for cancer (Bambace *et al.*, 2011; Radziwon-Balicka *et al.*, 2012). In this context, ASA could be a good candidate as our present data show that it completely suppress the angiogenic responses induced by releasates from thrombin-activated platelets. Interestingly, a recent analysis of 43 randomized trials of daily ASA administration for the primary and secondary prevention of vascular diseases showed a significant 12% reduction in the risk of cancer death suggesting that the inhibition of platelet activation may mediate both, the cardioprotective and cancer-preventive effect of low dose ASA (Rothwell *et al.*, 2012; Thun *et al.*, 2012). Moreover, the inhibition of several proangiogenic molecules might overcome the drawbacks of the current single-molecule blockade, which includes the fact that tumors may circumvent the inhibition of a single angiogenic protein by alternative expression of another angiogenic factor (Sennino *et al.*, 2012).

In conclusion, we demonstrate that although thrombin is capable of stimulating the secretion of both VEGF and endostatin, the effect of releasates from thrombin-stimulated platelets is clearly proangiogenic. This response is independent of VEGF action and it appears to be mainly due to the combined action of several intraplatelet proangiogenic molecules.

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REFERENCES

Albini A, Tosetti F, Li VW, Noonan DM, Li WW (2012). Cancer prevention by targeting angiogenesis. *Nat Rev Clin Oncol* **9**(9)**:** 498-509.

Alexander S, Harmar A, McGrath I (2011). New updated GRAC Fifth Edition with searchable online version Launch of new portal Guide to Pharmacology in association with NC-IUPHAR Transporter-Themed Issue. *Br J Pharmacol* **164**(7)**:** 1749-1750.

Arnaoutova I, George J, Kleinman HK, Benton G (2009). The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* **12**(3)**:** 267-274.

Bambace NM, Holmes CE (2011). The platelet contribution to cancer progression. *J Thromb Haemost* **9**(2)**:** 237-249.

Bambace NM, Levis JE, Holmes CE (2010). The effect of P2Y-mediated platelet activation on the release of VEGF and endostatin from platelets. *Platelets* **21**(2)**:** 85-93.

Battinelli EM, Markens BA, Italiano JE, Jr. (2011). Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis. *Blood* **118**(5)**:** 1359-1369.

Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R, Arthur HM (2006). Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism. *FASEB J* **20**(12)**:** 2009-2016.

Carmeliet P (2005). Angiogenesis in life, disease and medicine. *Nature* **438**(7070)**:** 932-936.

Coppinger JA, O'Connor R, Wynne K, Flanagan M, Sullivan M, Maguire PB*, et al.* (2007). Moderation of the platelet releasate response by aspirin. *Blood* **109**(11)**:** 4786-4792.

Chatterjee M, Huang Z, Zhang W, Jiang L, Hultenby K, Zhu L*, et al.* (2011). Distinct platelet packaging, release, and surface expression of proangiogenic and antiangiogenic factors on different platelet stimuli. *Blood* **117**(14)**:** 3907-3911.

Cho MS, Bottsford-Miller J, Vasquez HG, Stone R, Zand B, Kroll MH*, et al.* (2012). Platelets increase the proliferation of ovarian cancer cells. *Blood*.

Etulain J, Lapponi MJ, Patrucchi SJ, Romaniuk MA, Benzadon R, Klement GL*, et al.* (2011). Hyperthermia inhibits platelet hemostatic functions and selectively regulates the release of alpha-granule proteins. *J Thromb Haemost* **9**(8)**:** 1562-1571.

Etulain J, Negrotto S, Carestia A, Pozner RG, Romaniuk MA, D'Atri LP*, et al.* (2012). Acidosis downregulates platelet haemostatic functions and promotes neutrophil proinflammatory responses mediated by platelets. *Thromb Haemost* **107**(1)**:** 99-110.

Han N, Jin K, He K, Cao J, Teng L (2011). Protease-activated receptors in cancer: A systematic review. *Oncol Lett* **2**(4)**:** 599-608.

Ho-Tin-Noe B, Goerge T, Cifuni SM, Duerschmied D, Wagner DD (2008). Platelet granule secretion continuously prevents intratumor hemorrhage. *Cancer Res* **68**(16)**:** 6851-6858.

Italiano JE, Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S*, et al.* (2008). Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* **111**(3)**:** 1227-1233.

Jonnalagadda D, Izu LT, Whiteheart SW (2012). Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. *Blood* **120**(26)**:** 5209-5216.

Kamykowski J, Carlton P, Sehgal S, Storrie B (2011). Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet alpha-granules. *Blood* **118**(5)**:** 1370-1373.

Kim KW, Moretti L, Lu B (2008). M867, a novel selective inhibitor of caspase-3 enhances cell death and extends tumor growth delay in irradiated lung cancer models. *PLoS One* **3**(5)**:** e2275.

Klement GL, Yip TT, Cassiola F, Kikuchi L, Cervi D, Podust V*, et al.* (2009). Platelets actively sequester angiogenesis regulators. *Blood* **113**(12)**:** 2835-2842.

Li Z, Delaney MK, O'Brien KA, Du X (2010). Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* **30**(12)**:** 2341-2349.

Ma L, Perini R, McKnight W, Dicay M, Klein A, Hollenberg MD*, et al.* (2005). Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. *Proc Natl Acad Sci U S A* **102**(1)**:** 216-220.

Maloney JP, Silliman CC, Ambruso DR, Wang J, Tuder RM, Voelkel NF (1998). In vitro release of vascular endothelial growth factor during platelet aggregation. *Am J Physiol* **275**(3 Pt 2)**:** H1054-1061.

Martorell L, Martinez-Gonzalez J, Rodriguez C, Gentile M, Calvayrac O, Badimon L (2008). Thrombin and protease-activated receptors (PARs) in atherothrombosis. *Thromb Haemost* **99**(2)**:** 305-315.

Mohle R, Green D, Moore MA, Nachman RL, Rafii S (1997). Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. *Proc Natl Acad Sci U S A* **94**(2)**:** 663-668.

Moser M (2008). [The role of thrombin in angiogenesis]. *Hamostaseologie* **28**(4)**:** 189-194.

Negrotto S, Malaver E, Alvarez ME, Pacienza N, D'Atri LP, Pozner RG*, et al.* (2006). Aspirin and salicylate suppress polymorphonuclear apoptosis delay mediated by proinflammatory stimuli. *J Pharmacol Exp Ther* **319**(2)**:** 972-979.

Patzelt J, Langer HF (2012). Platelets in Angiogenesis. *Curr Vasc Pharmacol* **10**(5)**:** 570-577.

Peterson JE, Zurakowski D, Italiano JE, Jr., Michel LV, Connors S, Oenick M*, et al.* (2012). VEGF, PF4 and PDGF are elevated in platelets of colorectal cancer patients. *Angiogenesis* **15**(2)**:** 265-273.

Peterson JE, Zurakowski D, Italiano JE, Jr., Michel LV, Fox L, Klement GL*, et al.* (2010). Normal ranges of angiogenesis regulatory proteins in human platelets. *Am J Hematol* **85**(7)**:** 487-493.

Pietramaggiori G, Scherer SS, Cervi D, Klement G, Orgill DP (2008). Tumors stimulate platelet delivery of angiogenic factors in vivo: an unexpected benefit. *Am J Pathol* **173**(6)**:** 1609-1616.

Pipili-Synetos E, Papadimitriou E, Maragoudakis ME (1998). Evidence that platelets promote tube formation by endothelial cells on matrigel. *Br J Pharmacol* **125**(6)**:** 1252-1257.

Radziwon-Balicka A, Moncada de la Rosa C, Jurasz P (2012). Platelet-associated angiogenesis regulating factors: a pharmacological perspective. *Can J Physiol Pharmacol* **90**(6)**:** 679-688.

Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z (2012). Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* **379**(9826)**:** 1591-1601.

Sabrkhany S, Griffioen AW, Oude Egbrink MG (2011). The role of blood platelets in tumor angiogenesis. *Biochim Biophys Acta* **1815**(2)**:** 189-196.

Sehgal S, Storrie B (2007). Evidence that differential packaging of the major platelet granule proteins von Willebrand factor and fibrinogen can support their differential release. *J Thromb Haemost* **5**(10)**:** 2009-2016.

Sennino B, McDonald DM (2012). Controlling escape from angiogenesis inhibitors. *Nat Rev Cancer* **12**(10)**:** 699-709.

Thun MJ, Jacobs EJ, Patrono C (2012). The role of aspirin in cancer prevention. *Nat Rev Clin Oncol* **9**(5)**:** 259-267.

van Nispen tot Pannerden H, de Haas F, Geerts W, Posthuma G, van Dijk S, Heijnen HF (2010). The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes. *Blood* **116**(7)**:** 1147-1156.

Wang Y, Wei X, Xiao X, Hui R, Card JW, Carey MA*, et al.* (2005). Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. *J Pharmacol Exp Ther* **314**(2)**:** 522-532.

Weis SM, Cheresh DA (2011). Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* **17**(11)**:** 1359-1370.

Yu P, Yu DM, Qi JC, Wang J, Zhang QM, Zhang JY*, et al.* (2006). [High D-glucose alters PI3K and Akt signaling and leads to endothelial cell migration, proliferation and angiogenesis dysfunction]. *Zhonghua Yi Xue Za Zhi* **86**(48)**:** 3425-3430.

FIGURE LEGENDS

Figure 1. Thrombin induces the secretion of intraplatelet VEGF, endostatin and ATP, and TXB_2 generation. WPs were stimulated with different concentration of thrombin (Thr) for 5 min under stirring and non-stirring conditions. (A) VEGF and endostatin levels in the supernatants were quantified by ELISA (n=4). (B) and (C) WPs were stimulated with thrombin (0.05 U ml^{-1}) for 5 min under stirring and non-stirring conditions. (B) ATP release was measured by using a lumi-aggregometer and (C) TXB_2 levels were determined by ELISA (n=4, **P* < 0.05 vs. unstimulated, **#P < 0.05 vs. Stirring**).

Figure 2. The releasate from platelets activated with thrombin trigger proangiogenic processes that are primarily a VEGF-independent response. HMEC-1 were preincubated in 48-well plates without (A) and (B) or with (C) and (D) anti-VEGF receptor antibody (anti-VEGFR, 2 μg ml^{-1}) for 30 min. Cells were then stimulated with recombinant VEGF (rVEGF, 20 ng ml^{-1}) (positive control) or with platelet (Plt) supernatants from unstimulated (Unst) or thrombin-stimulated (0.05 or 1 U ml^{-1}) platelets after 18 h. (A) and (C) Endothelial proliferation was determined by addition of MTT reagent. The reaction was stopped and the absorbance at 570 nm was measured. (B) and (D) Tube formation in the matrigel-coated wells was analyzed under an inverted light microscope, and the number of branch points in four non-overlapping fields was determined. The scale bar is 200 μm in size (n=5, * *P* < 0.05 vs. unstimulated, # *P* < 0.05 vs. without anti-VEGFR).

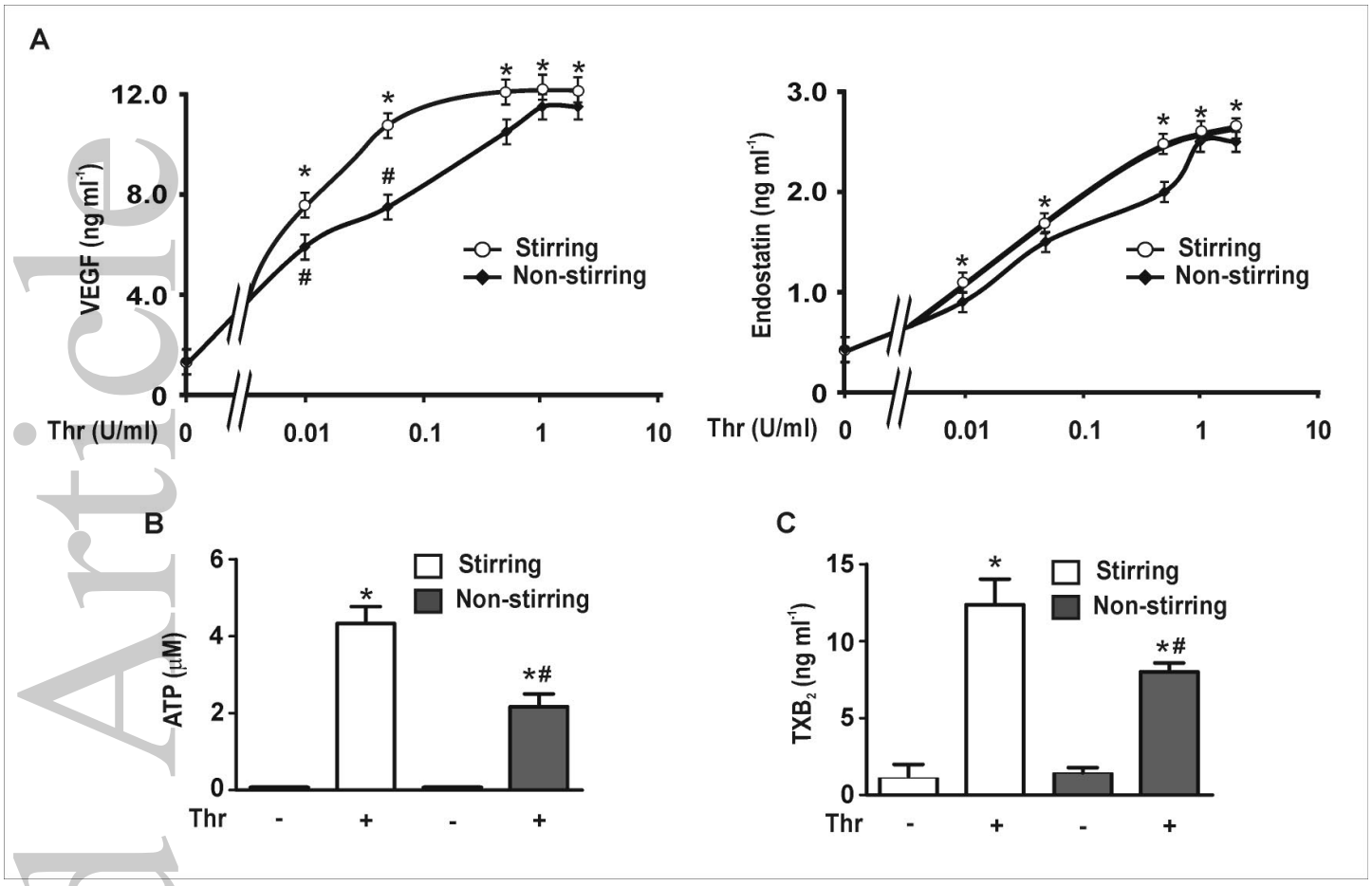
Figure 3. The levels of angiogenic proteins in the releasate of thrombin-activated platelets. WPs were stimulated with thrombin (0.05 U ml^{-1}) for 5 min. The release of angiogenic regulators was measured using commercial array kits according to the manufacturer's instructions. The thrombin-induced release of each molecule is expressed as the fold increase over unstimulated controls (n=2).

Figure 4. Signaling pathways are involved in platelet-mediated angiogenesis. WPs were incubated with or without the inhibitors of COX-1 (ASA, 500 μM or Indomethacin (Indo), 30 μM), PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10 μM) pathways for 30 min. Platelets were then stimulated with thrombin (0.05 **or 1** U ml^{-1}) for 5 min. Platelet supernatants were obtained and used to induce angiogenic responses. (A) Endothelial proliferation was determined by the addition of MTT reagent. The reaction was stopped and the absorbance at 570 nm was measured. (B) Tube formation in the matrigel-coated wells was analyzed under an inverted light microscope, and the number of branch points in four non-overlapping fields was determined. The results were expressed as the percentage of inhibition vs thrombin (without inhibitor) (n=5, # $P < 0.05$ vs. without inhibitor). (C) Schematic representation of the inhibited platelet pathways.

Figure 5. The signaling pathways involved in the secretion pattern of thrombin-triggered angiogenesis-modulating platelet products. WPs were incubated with or without the inhibitors of COX-1 (ASA, 500 μM), PKC (Gö6983, 1 μM), p38 (SB203580, 25 μM), ERK1/2 (U0126, 10 μM), Src kinases (PP1, 5 μM), or PI3K/Akt (Ly-294002, 10

μM) pathways for 30 min. Platelets were then stimulated with thrombin (0.05 U ml^{-1}) for 5 min and supernatants were obtained. The release of angiogenic regulators was measured by using commercial array kits. The graphs demonstrate (A) the mean of fluorescence intensity (MFI) of each indicated molecule, and (B) thrombin-induced VEGF release measured by ELISA (n=3) or array (n=2). (C) P-selectin exposure was detected by flow cytometry (n=5) and (D) ATP release was measured by using a Lumi-aggregometer. The results were expressed as the percentage of inhibition vs thrombin (without inhibitor) (n=5, # $P < 0.05$ vs. without inhibitor).

The authors declare no conflict of interest.



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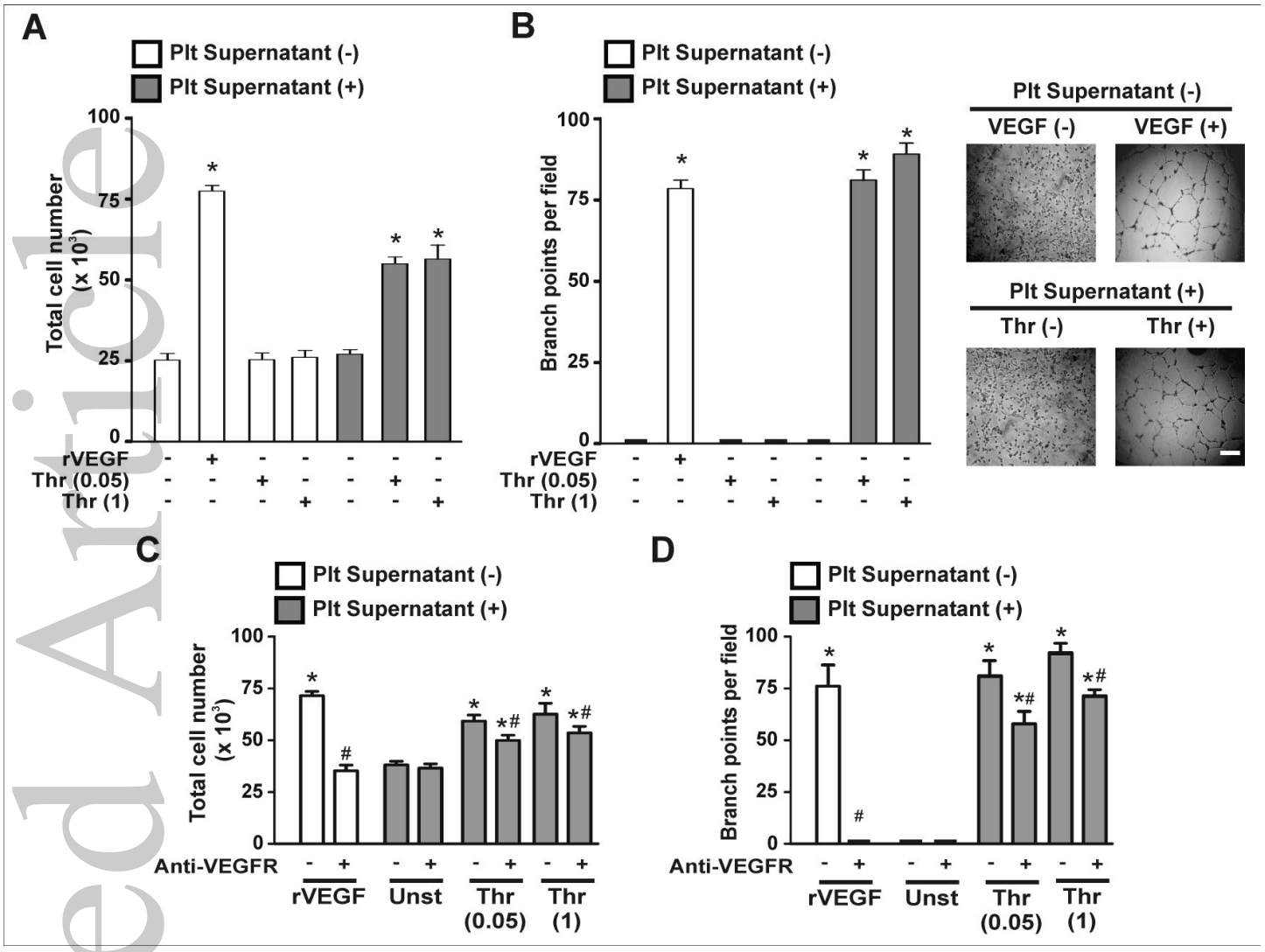

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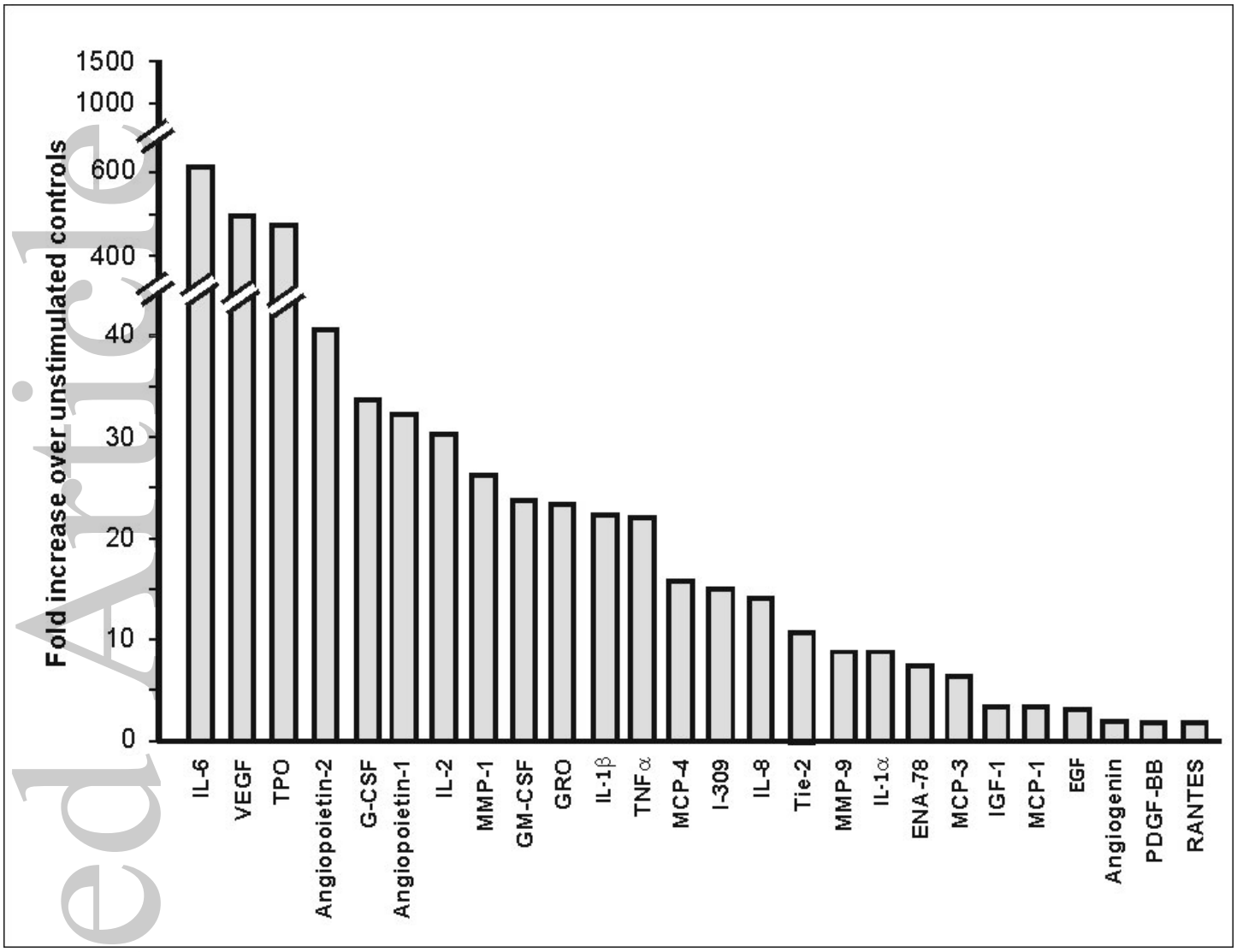

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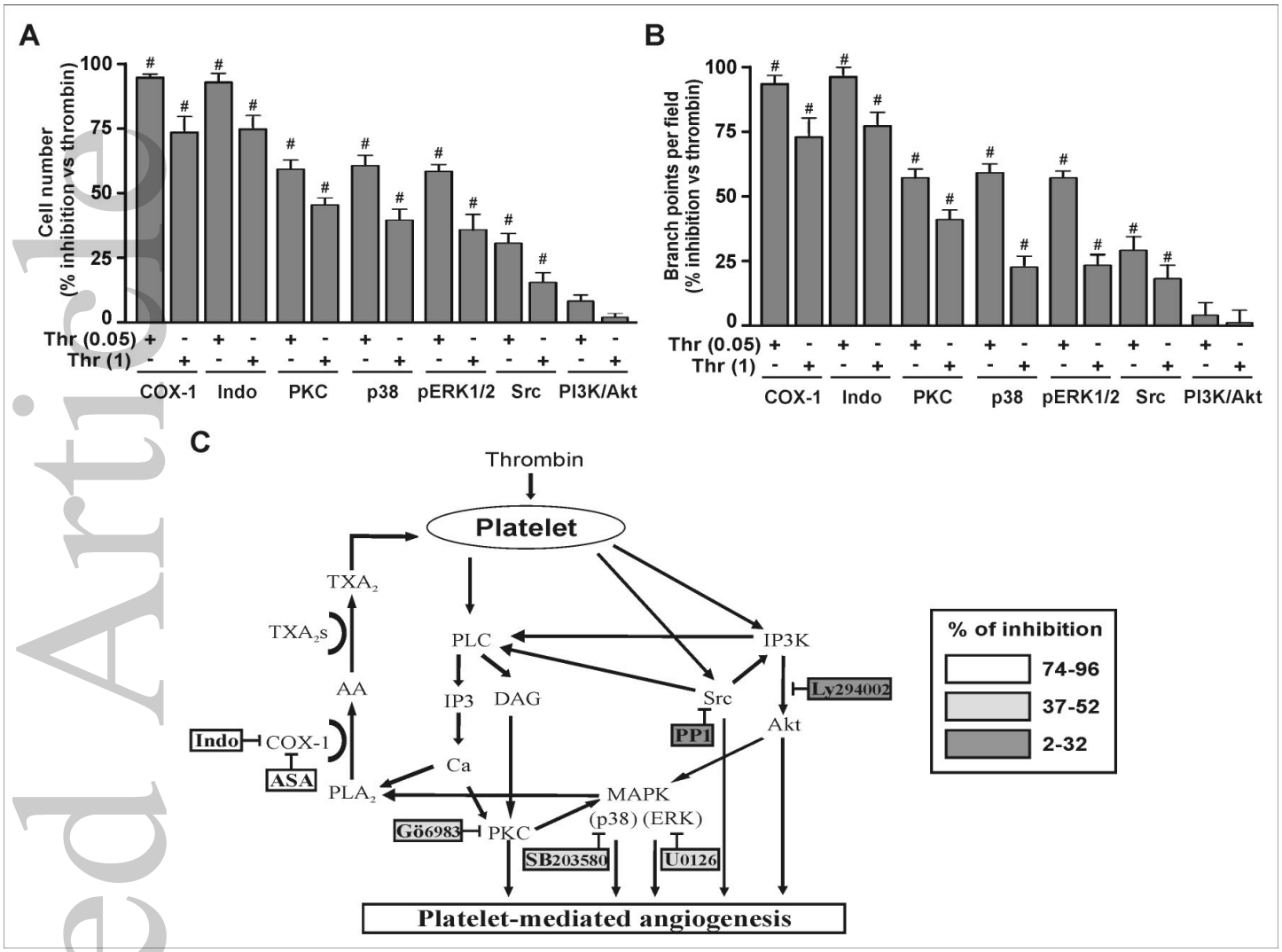

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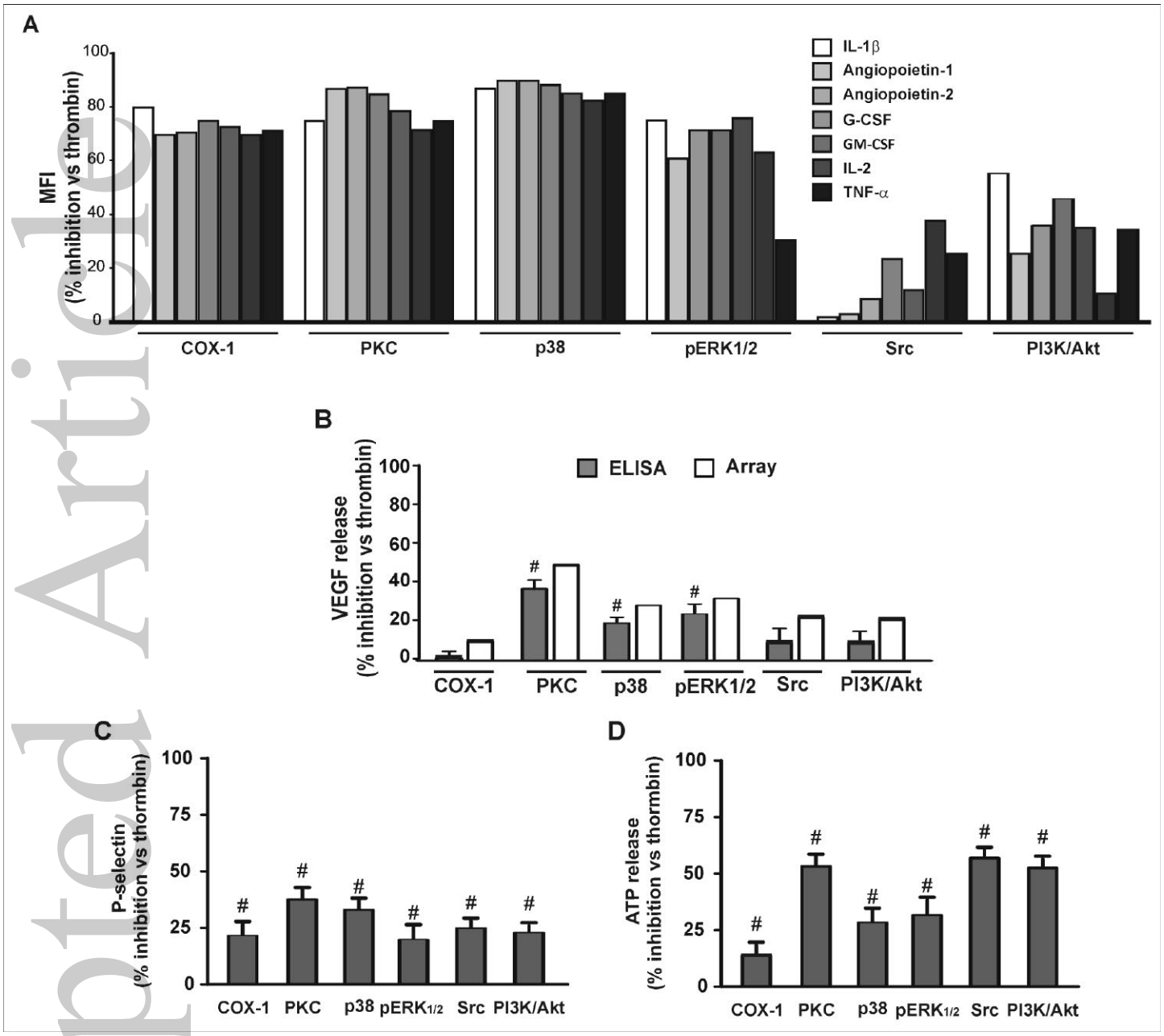

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