Platelet-associated Angiogenesis Regulating Factors: A Pharmacological Perspective

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Abstract

Platelets in addition to maintaining hemostasis also stimulate angiogenesis by generating and releasing upon activation factors that promote the growth of new blood vessels. To date, at least twenty angiogenesis regulating factors have been identified in platelets including both promoters and inhibitors. Platelet-derived angiogenesis regulators promote angiogenesis during wound healing, tumor growth, and in response to ischemia. Within platelets, angiogenesis regulators are primarily stored in α-granules, but are also found in the cytosol or derived from membrane lipids. Their release can be inhibited pharmacologically by anti-platelet agents, which consequently suppress platelet-stimulated angiogenesis. Several years ago our research group identified that platelets generate the angiogenesis inhibitor angiostatin independent of the activation state of platelets, and that platelet-derived angiostatin serves to limit the angiogenesis-stimulating effects of platelets. In this review, we summarize current knowledge of platelet-associated angiogenesis regulators, how they impact angiogenesis, and how they are pharmacologically controlled.

Key words

Platelets, angiogenesis, hemostasis, thrombosis, endothelial cells, angiogenesis regulators, angiostatin, vascular endothelial growth factor, alpha-granules

Blood Platelets

Platelets are anucleate cell fragments (approx. 1 - 4 μm in diameter) which maintain hemostasis (Bizzozero, 1882). They are generated from megakaryocytes, and if not used up in hemostatic reactions, have an approximate 10 day lifespan within the human circulation before being cleared by the liver and spleen (Dowling et al. 2010; Leeksma & Cohen 1955). In their inactivated state platelets are discoid in shape; however, in response to agonists that are exposed to flowing blood or generated upon vascular injury (eg. collagen, thrombin), they undergo dynamic cytoskeletal rearrangement extending pseudopodia in multiple directions (Finch 1969). In addition to lacking nuclei, platelets have a number of characteristic features. Their outer surface membrane is rich in glycoproteins that largely make up the receptors for stimuli triggering platelet activation, adhesion, and aggregation. Receptor stimulation by agonists activates signalling pathways responsible for mediating platelet adhesion and aggregation reactions (Levy-Toledano 1999). The final step in the signalling cascade results in a conformational change of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) leading to its activation and binding of fibrinogen (and/or other adhesion proteins such as vWF and fibronectin) (Ni et al. 2003) forming bridges between aggregating platelets as well as stabilizing initial platelet adhesion (Savage et al. 1998). Platelets also contain many organelles including mitochondria, lysosomes, dense bodies, and alpha-granules. Platelet dense bodies also known as dense or δ-granules contain ADP, serotonin, and noradrenaline, which are secreted from the platelet upon aggregation. These secreted products along with thromboxane A_2 generated from platelet membrane arachidonic acid and matrix metalloproteinase-2 released from the platelet cytosol help mediate and amplify aggregation by acting in an autocrine and paracrine manner recruiting other platelets to the aggregate. The resulting platelet plug serves to seal an injured blood vessel

and prevent blood loss. To prevent uncontrollable aggregation endogenous systems exist to stop these reactions. Platelets generate and/or release nitric oxide (NO) (Radomski et al. 1990), MMP-9 (Jurasz et al. 2002), and tissue inhibitor of matrix metalloproteines-4 (TIMP-4) (Radomski et al. 2002), which inhibit aggregation, while the endothelium generates the disaggregating factor prostacyclin (PGI_2) (Radomski et al. 1987a) and NO (Radomski et al. 1987b). Hence, a platelet balance exists that maintains hemostasis. A pathological extension of hemostasis is thrombosis during which the hemostatic system is activated and platelet plugs are formed in absence of vascular injury.

In addition to maintaining hemostasis platelets help coordinate a number of important non-hemostatic responses to injury including inflammation (Klinger, 1997), immunity (Semple et al. 2011; Vieira-de-Abreu et al. 2011), and angiogenesis (Browder et al. 2000; Jurasz et al. 2003). Angiogenesis is of particular importance to the proper and timely healing of injured tissues, and the platelet influence on this physiological (and often pathological) response will be the focus of this review.

Platelets and Angiogenesis

Angiogenesis is the process by which new capillaries form from pre-existing blood vessels. It differs from vasculogenesis which is defined as the formation of a vascular plexus from endothelial cell precursors (angioblasts) that subsequently differentiate into endothelial cells (Conway et al. 2001); initially vasculogenesis referred to early vascular growth in the embryo. Mechanistically, new capillaries can arise from outgrowths (sprouting angiogenesis) of existing blood vessels or from blood vessel ingrowths (intussusceptive angiogenesis) caused by the insertion and extension of luminal pillars (Burri et al. 2004; Burri & Tarek 1990). Sprouting angiogenesis is primarily regulated by the local concentrations of angiogenesis promoting and

inhibiting factors, while intussuceptive angiogenesis is largely governed by local hemodynamic forces. Temporally sprouting angiogenesis occurs in two phases: initiation and stabilization. Factors such as vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF) and matrix metalloproteinases (MMPs) promote and mediate the initial phase of angiogenesis (Deryugina et al. 2010; Ferrara 2009; Pepper et al. 1992). This phase consists of the dissolution of the basement membrane and surrounding matrix of the parent vessel, followed by endothelial migration, proliferation, and lumen formation. Of particular importance during sprout initiation is activation of Notch receptor signalling within endothelial cells. The Notch signalling pathway determines which cells become sprout-leading tip cells and which cells become sprout base forming stalk cells (Thurston et al. 2007; Kuhnert et al. 2011). The Notch receptors consist of a family of four members of which Notch1 and 4 have been found to be expressed by the endothelium. Notch signalling can be activated by five ligands including Delta like ligand 1 (Dll1), Dll3, Dll4, Jagged1, and Jagged2. In response to VEGF, sprout forming tip cells increase their expression of Dll4 and activate Notch signalling in adjacent endothelial cells causing a down-regulation of VEGF receptor-2 (VEGFR-2) and the acquisition of the stalk cell phenotype (Patel-Hett and D'Amore 2011). This negative-feedback mechanism prevents excessive tip cell formation and allows for a directional, organized migratory response by endothelial cells toward a pro-angiogenic growth factor gradient (Phng and Gerhardt 2009). In addition, stalk cells express Jagged 1, which suppresses co-expressed Dll4 from activating Notch signalling in tip cells thereby allowing sustained expression of VEGFR at the angiogenic front (Benedito et al. 2009; Eilken and Adams 2010). The stabilization phase follows whereby endothelial cell proliferation stops, the basement membrane is reconstructed, and the capillaries are surrounded by pericytes. During this phase, factors such as angiopoietin-1 and platelet derived growth factor

(PDGF) are crucial to the maturation of newly formed vessels (Betsholtz et al. 2005; Fam et al. 2003; Suri et al. 1996). Conversely, endogenous negative regulators such, as thrombospondin-1 (TSP-1) and platelet factor 4 (PF4), block endothelial cell integrins and growth factor receptors interfering with migration and proliferation (Maione et al. 1990; Roberts 1996). Tissue inhibitors of metalloproteinases (TIMPs) inhibit basement membrane and extracellular matrix remodeling by MMPs (Schnaper et al. 1993), and angiostatin inhibits MMP release from endothelial cells (Jurasz et al. 2006). Many of the factors that regulate angiogenesis are either stored or generated by platelets and are released upon aggregation in response to injury.

Within adults, physiological angiogenesis takes place during wound healing, within the corpus luteum and endometrium during the female menstrual cycle (Demir et al. 2010; Girling et al. 2005), and during placentation (Huppertz et al. 2005). Pathologically, angiogenesis occurs in cancer (Hanahan1996), vascular retinopathy (Giuliari et al. 2010), rheumatoid arthritis (Szekanecz et al. 2009), atherosclerosis (Doyle et al. 2007), as well as other diseases (Folkman 2007). One of the earliest recognized roles for platelet-stimulated angiogenesis was discovered in the context of studying wound healing in the early 1980's (Knighton et al. 1982). Using a rabbit cornea assay, it was found that implantation of autologous thrombin-activated platelets stimulated angiogenesis *in vivo*. The angiogenesis-stimulating effect was dependent on the concentration of activated-platelets injected and was mediated by platelet-released factors as unactivated platelets or activated platelets subsequently washed with buffer failed to stimulate angiogenesis (Knighton et al. 1982). More recently, Wallace and colleagues have shown that platelets play a vital role in gastic ulcer healing. Thrombocytopenic rats have significantly impaired gastric ulcer healing and reduced angiogenesis of granulated tissue compared to control rats with normal platelet counts (Ma et al. 2001). A similar impairment in gastric ulcer healing

and depressed angiogenesis of granulated tissue was observed when rats were treated with ticlopidine an inhibitor of $P2Y_{12}$ and ADP-mediated platelet aggregation, again pointing to factors released upon aggregation as the primary mediators of platelet-stimulated angiogenesis.

One of the first studies recognizing that platelets may contribute to tumor angiogenesis was made in the late 1970's when it is found that tumor extracts in combination with platelets or their factors potentiated the growth rate of endothelial cells on collagen substratum (Schor et al. 1979). Similarly, serum with high VEGF concentrations derived from cancer patients stimulates human umbilical vein endothelial cell proliferation *in vitro* (Salgado et al. 1999*)* and indicates fast tumor growth rates in cancer patients (Dirix et al. 1996). Numerous studies have shown that cancer patient serum VEGF concentrations correlate with platelet counts (Salgado et al. 1999; Verheul et al. 1997), and that cancer patients have elevated serum and platelet VEGF levels (Caine et al. 2004; Salven et al. 1999). VEGF accumulates within platelets during tumor progression (Salgado et al. 2001), and there is a shift in the platelet VEGF to TSP-1 balance favouring VEGF in cancer patients (Gonzalez et al. 2004). Activated platelets have been identified in the tumor vasculature of patients with soft tissue sarcomas suggesting platelets contribute to tumor angiogenesis (Verheul et al. 2000). Studies in experimental models of tumor growth have yielded similar results. Platelets of tumor-bearing mice have higher intracellular levels of VEGF, bFGF, PDGF, and even TSP-1 and PF-4 during early tumor growth (Cervi et al. 2008; Klement et al. 2009; Zaslavsky et al. 2010). Circulating platelets can take up angiogenesis regulators from tumors (Klement et al. 2009), or their synthesis can be up-regulated in bone marrow megakaryocytes suggesting cross-talk between the tumor and the hematopoietic system (Zaslavsky et al. 2010). Platelets of tumor-bearing mice stimulate angiogenesis to a greater extent than platelets from non-tumor-bearing mice (Pietramaggiori et al. 2008), while various

cancer cell lines such as HT-1080, A549, and MCF-7 stimulate release of angiogenesis regulators such as MMP-2, VEGF, and angiostatin from platelets (Battinelli et al. 2011; Jurasz et al. 2003; Jurasz et al. 2001). In addition to platelet-stored factors, platelet membrane and granule-derived microvesicles and exosomes stimulate expression of angiogenic genes in A549 cells and tube formation by endothelial cells, suggesting an important role for platelet-derived lipids in promoting angiogenesis (Janowska-Wieczorek et al. 2005; English et al. 2000; Kim et al. 2004). In addition to promoting angiogenesis, platelets preferentially adhere to angiogenic vessels (Kisucka et al. 2006) and stabilize tumor vasculature preventing intratumoral hemorrhage by secreting angiopoietin-1 and serotonin, which reduce permeability of tumor vessels (Ho-Tin-Noe et al. 2008). Targeting platelet-stimulated angiogenesis may be a novel approach to disrupting tumor angiogenesis. In fact, experimental studies have shown that blockade of platelet GPIIb/IIIa with the anti-platelet antibodies 10E5 and Abciximab inhibited VEGF release from tumor-activated platelets, platelet-stimulated endothelial cell sprouting, and experimental metastasis (Amirkhosravi et al. 1999; Trikha et al. 2002).

Platelets not only promote tumor angiogenesis, but also stimulate revascularization in response to ischemia. Implantation of platelets (or platelets and peripheral blood mononuclear cells) into the ischemic hind limbs of rats stimulates angiogenesis within the adductor muscles by delivering factors such as VEGF, bFGF, PDGF, and TGFβ (Iba et al. 2002). These findings are supported by studies in thrombopoietin (TPO-/-) null and TPO-receptor (c-mpl-/-) null mice. TPO-/- and c-mpl-/- mice, which are thrombocytopenic, have an impaired endogenous angiogenic response to acute hind limb ischemia (Amano et al. 2005). The delivery of angiogenesis promoting factors can also be accomplished by platelet-derived microparticles (PMPs), which when injected into the ischemic heart muscle of rats following left anterior

descending coronary artery ligation, stimulate capillary growth in the ischemic myocardium (Brill et al. 2005). Like with whole intact platelets, PMPs likely deliver VEGF, bFGF, and PDGF to sprouting endothelial cells since antibody-dependent neutralization of bFGF, PDGF and inhibition of VEGFR tyrosine phosphorylation reduces PMP-induced angiogenesis within the aortic ring model (Brill et al. 2005; Shai and Varon 2010). This capacity for PMPs to stimulate angiogenesis may in part explain the angiogenesis promoting effects of early outgrowth endothelial progenitor cells (EPCs). Platelets have been shown to recruit and aid in the differentiation of EPCs to mature endothelial cells in culture (Langer et al. 2006; Massberg et al. 2006). However, recent proteomic analysis of EPCs cultured from peripheral blood mononuclear cells has revealed that mononuclear cells take up PMPs which are generated from platelets that are invariably isolated along with mononuclear cells (Prokopi et al., 2009). Platelets share many of the same markers as endothelial cells such as CD31 and von Willebrand factor (vWF), and PMPs transfer these markers to cultured mononuclear cells (Prokopi and Mayr 2011). The removal of PMPs or their antagonism by platelet glycoprotein IIb/IIIa inhibitors attenuates the capillary network promoting effects of EPC conditioned medium (Prokopi et al. 2009; Prokopi & Mayr 2011). It is likely that PMPs in addition to transferring in-common endothelial cell markers also transfer some of their angiogenic potential in the form of platelet-associated angiogenesis regulating factors to cultured endothelial progenitor cells/monocytes.

Platelets are also increasingly being shown to participate in vasculogenesis/angiogenesis. Platelet α-granule-secreted factors promote bone marrow-derived cell recruitment to sites of neovascularisation in ischemic limbs and tumors (Feng et al. 2011). The role of platelets in vasculogenesis needs to be explored further, but interestingly it has already been shown that platelets play a morphogenetic role separating blood and lymphatic vasculatures within the

embryo (Carramolino et al. 2010). A mechanism has been proposed whereby platelets activated by lymphoendothelial-specific molecules at the lymphatic-venous interface form a barrier preventing connections between lymphatic and venous vasculatures (Carramolino et al. 2010). In addition, it should be noted that VEGF-C, which is required for lymphangiogenesis, is released from activated platelets (Wartiovaara et al. 1998).

Platelet-associated Angiogenesis Regulating Factors

Circulating platelets contain an abundant array of positive and negative angiogenesis regulators (English et al. 2000; Mohle et al. 1997; Kaplan et al. 1997; Sawicki et al. 1997; Li et al. 2001; Assoian et al. 1983) (Table 1). These angiogenesis regulators are found in various platelet compartments including platelet membranes, cytosol, and granules. Sphingosine-1 phosphate (S1-P) is a lipid second messenger derived from membrane sphingosine. S1-P is rapidly formed and released by platelets in responses to activation by collagen and thrombin (Yatomi et al. 1995). It potently stimulates endothelial cell chemotactic motility as well as serving as a mitogen (English et al. 2001; English et al. 2000; Lee et al. 1999). Within their cytoplasm platelets store MMP-2 which translocates to the platelet surface membrane and extracellular space upon aggregation (Sawicki et al. 1998). While platelet MMP-2 has been studied primarily for its aggregation mediating effects (Jurasz et al. 2002; Santos-Martínez et al. 2008; Sawicki et al. 1997), it also has the capacity to degrade a blood vessel's endothelial cell anchoring basement membrane, thus facilitating endothelial cell migration (Schnaper et al. 1993).

The most widely studied platelet storage organelle of angiogenesis regulators is the α-granule. To date the majority of the protein angiogenesis regulators have been confirmed to be stored in platelet α-granules including: VEGF, bFGF, PDGF, EGF, IGF, TSP-1, endostatin, PF4,

TGFβ1; while angiostatin is likely stored in α-granules since plasminogen/angiostatin immunoreactivity localizes to the α-granule (Italiano et al. 2008; Gerrard et al. 1980; Kaplan et al. 1979; Karey et al. 1989; Mitjavila et al. 1988; Pesonen et al. 1989; Radomski et al. 2002; Salgado et al. 2001). The platelet ability to differentially release angiogenesis stimulating and inhibiting factors was first identified by Ma and colleagues who investigated VEGF and endostatin release from platelets in response to activation of proteinase-activated receptors 1 and 4 (PAR1 and PAR4) (Ma et al. 2005) the receptors for thrombin. Stimulation of platelets with a selective PAR1 activating peptide resulted in the release of VEGF, while stimulation of PAR4 caused endostatin to be released. Similar results have been obtained by Italiano and Klement who importantly further showed that platelet angiogenesis stimulators VEGF and bFGF are segregated into separate α-granules from angiogenesis inhibitors TSP-1 and endostatin (Italiano et al. 2008). More recently it has been demonstrated that the ADP receptors, $P2Y_1$ and $P2Y_{12}$ are also involved in the regulation of angiogenic protein exocytosis although these pathways seem to lead to less release of VEGF than PAR-mediated activation (Bambace et al. 2010). Platelet VEGF release can be abolished pharmacologically by selectively inhibiting both $P2Y_1$ and $P2Y_{12}$ receptors (Bambace et al. 2010; Battinelli et al. 2011), and hydrolysis of ADP by apyrase attenuates platelet-induced angiogenesis within the aortic ring angiogenesis model (Brill et al. 2004). Similarly, acetylsalicylic acid has been shown to reduce VEGF release from platelets and platelet-stimulated endothelial cell migration and capillary tube formation on Matrigel (Battinelli et al. 2011).

The source(s) of platelet-associated angiogenesis regulators remain under active investigation, but platelets can acquire them in a number of ways including via uptake from the circulation, inheritance from their parent cells megakaryocytes, or via active synthesis. Platelets

incubated with increasing concentrations of endostatin or bFGF take up the proteins in a dose-dependent manner, and injection of matrigel incorporating ^{125}I-labeled VEGF into mice has shown that after three days the VEGF preferentially accumulates in platelets over plasma or other tissues (Klement et al. 2009). This ability to sequester angiogenesis regulators, particularly if their source is a tumor, makes platelets attractive candidates as tumor angiogenesis biomarkers (Caine et al. 2004; Peterson et al. 2010). In addition, megakaryocytes transport α-granules containing VEGF, bFGF, endostatin, or TSP-1 into platelets during their formation (Italiano et al. 2008). This source is likely to account for many of the angiogenesis regulators found in platelets under physiological conditions. Finally, platelets can actively synthesize both angiogenesis stimulators and inhibitors. In addition to S1-P which is generated during the catabolism of sphingosine upon platelet activation, a recent study has shown that thrombin-stimulated platelets generate deoxyribose 1-phosphate (dRP) as a result of uridine phosphorylase-mediated phosphorolytic degradation of deoxynucleosides (Pula et al. 2010). Platelet-derived dRP stimulates endothelial cell migration and angiogenesis within the chick chorioallantoic membrane assay. Conversely, platelets also generate the angiogenesis inhibitor angiostatin, but do so constitutively independent of their activation state (Jurasz et al. 2006). Platelet-derived angiostatin has been the research focus of our group for the last number of years.

Angiostatin: An Angiogenesis Inhibitor Actively Generated by Platelets

Angiostatin was first discovered in the laboratory of the late Dr. Folkman in a Lewis Lung carcinoma (LLC) model of concomitant resistance (O'Reilly et al. 1994). It was identified in the serum and urine of mice with primary LLC tumors and found to be a cleavage product of plasminogen containing the first four of five plasminogen subunits named kringles (K1-4) (Soff

2000). Proteolysis of plasminogen into angiostatin can be accomplished by two classes of endopeptidases, the serine and matrix metalloproteinases (MMPs). Serine proteases such as elastase (O'Reilly et al. 1994), prostate specific antigen (Heidtmann et al. 1999), tissue-plasminogen activator (tPA), urokinase plasminogen activator (uPA) (Gately et al. 1997) and MMPs such as MMP-2 (O'Reilly et al. 1999), MMP-3 (Lijnen et al. 1998), MMP-7, MMP-9 (Patterson et al. 1997), and MMP-12 (Dong et al. 1997) have been shown to generate angiostatin K1-4 or other angiostatins with different numbers of kringles (K1-3, K1-4.5, K1-5). Initially, angiostatin was found to be generated by cancer cells (Gately et al. 1996; Stathakis et al. 1997; Westphal et al. 2000) and by macrophages during inflammation (Falcone et al. 1998); while angiostatin K1-3 can be generated by activated human neutrophils (Scapini et al. 2002). However, in mid 2000 while studying platelet MMP function (Jurasz et al. 2002; Jurasz et al. 2001; Sawicki et al. 1997) our research group postulated that human platelets may generate angiostatin. Our hypothesis was based on the knowledge that platelets contain both catalysts (MMPs and plasminogen activators) and substrate (plasminogen in their α-granules) for angiostatin generation. Further, platelet proteases are activated on the platelet surface membrane and have direct access to plasminogen in the circulation (Endresen et al. 1985; Miles et al. 1985; Sawicki et al. 1998). Our investigations found that platelets store angiostatin containing kringles 1-4 and release it in functional form upon aggregation (Jurasz et al. 2003). To determine if platelets can generate angiostatin *de novo*, a pharmacological approach was utilized focusing on the platelet surface membrane as the catalytic site for angiostatin production. Somewhat surprising to us at the time was the finding that MMPs are not involved in platelet angiostatin generation, since o-phenanthroline a broad-spectrum MMP inhibitor failed to suppress angiostatin generation by isolated platelet membranes (Jurasz et al. 2006). However, broad-

spectrum serine protease inhibitors aprotinin and leupeptin efficiently inhibited platelet angiostatin production. Using more specific inhibitors we identified that platelets generate angiostatin in an uPA-dependent manner (Figure 1). Moreover, platelets do not need to be in their activated state to generate angiostatin. We found that resting intact human platelets convert physiological concentrations of plasminogen to 20-25 μg/ml of angiostatin within 1 hour. Interestingly, human plasma concentrations of angiostatin K1-4 are approximately 30 μg/ml suggesting (Jurasz et al. 2010b), that in absence of cancer or inflammation, platelets are likely the primary source of circulating angiostatin K1-4 which is the predominant angiostatin in human plasma (Figure 2). This ability of platelets to constitutively generate large amounts of angiostatin separates it from other platelet-derived angiogenesis regulators that are either inherited from megakaryocytes or scavenged in the circulation. We propose that physiologically angiostatin generated by circulating platelets serves to help keep the endothelium quiescent and upon release from aggregating platelets serves to counterbalance the net angiogenesis stimulatory effects of platelets. Indeed, we have found that although platelet releasates stimulate formation of capillary-like structures by endothelial cells on extracellular matrix, inhibition of angiostatin in platelet releasates or its generation by platelets further promotes capillary-like structure formation (Jurasz et al. 2003; Jurasz et al. 2006). The role of platelet-derived angiostatin under pathological conditions still largely needs to be determined. However, a preliminary study identified high elevated levels of angiostatin in platelets derived from patients with idiopathic pulmonary arterial hypertension (iPAH) (Jurasz et al. 2010b). IPAH is characterized by a loss of pre-capillary arterials (Jurasz et al. 2010a) and pulmonary thrombosis (Johnson et al. 2006). Whether platelet-derived angiostatin contributes to loss of pre-capillary arterials in iPAH still needs to be determined; however, over-expression of angiostatin in the

lung aggravates PAH in the chronically hypoxic mouse (Pascaud et al. 2003). Similarly, whether platelet-derived angiostatin plays a pathogenic or therapeutic role in other diseases awaits further investigation.

Summary

Platelets have long been known to be primary mediators of thrombosis and hemostasis. Recently, they have been recognized as important contributors to angiogenesis as a consequence of the many angiogenesis regulating factors that are generated by and found within platelets. Platelet-associated angiogenesis regulators have the capacity to stimulate every stage of angiogenesis including endothelial cell migration, proliferation, differentiation into capillary-like structures, stabilization of newly formed vessels, and even recruitment of bone marrow-derived cells to sites of neo-angiogenesis. Further work will be required to elucidate how the platelet capacity for differential spatial and temporal release of angiogenesis regulators impacts tip and stalk cell selection during sprouting. No doubt pharmacology which has been used extensively to study platelet-stimulated angiogenesis will continue to play a major role in many future platelet-angiogenesis investigations. Moreover, pharmacological interventions that manipulate the generation and release of platelet-associated angiogenesis regulators have the potential to become important therapeutics in the treatment of angiogenesis-dependent diseases.

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Figure Legends

Figure 1. Schematic of platelet angiostatin generation. Platelets utilize plasma plasminogen and plasminogen derived from platelet α-granules to generate angiostatin on their surface membranes in a uPA-dependent manner via a plasmin. The generated angiostatin is released into the circulation and/or taken up into α-granules.

Figure 2. Immunoblot demonstrating that angiostatin K1-4, which has been shown to be generated by platelets, is the predominant angiostatin form in human plasma. Blood was drawn from healthy volunteers into trisodium citrate. Platelet-poor plasma (PPP) samples were isolated by first centrifuging whole blood for 20 minutes at 250 g in the presence of prostacyclin (200 nM) to obtain platelet-rich plasma (PRP). Subsequently, PRP was centrifuged at 900 g for 10 minutes in the presence of prostacyclin (800 nM) to obtain PPP. Platelet-poor plasma samples (2 μl) were subject to 12% SDS-PAGE and immunoblot was performed as described previously (Jurasz et al. 2003; Jurasz et al. 2006; Jurasz et al. 2010). Anti-angiostatin affinity purified polyclonal antibody (AF226) was obtained from R&D Systems (Minneapolis, MN, USA). Human angiostatin K1-4 protein was obtained from Oncogene (San Diego, CA, USA). Human plasminogen was obtained from Sigma (Oakville, ON, Canada). Angiostatin K1-4 (Ang K1-4), plasminogen (Plg).

Table 1: A list of platelet-associated angiogenesis regulating factors

Platelet-associated angiogenesis stimulators	Platelet-associated angiogenesis inhibitors
Vascular endothelial growth factor (VEGF)	Angiostatin
Basic fibroblast growth factor	Endostatin
Platelet-derived growth factor (PDGF)	Platelet factor 4 (PF4)
Epidermal growth factor (EGF)	Plasminogen activator inhibitor-1 (PAI-1)
Hepatocyte growth factor (HGF)	Transforming growth factor-β (TGF-β)
Insulin-like growth factor 1 and 2 (IGF-1 and IGF-2)	Thrombospondin-1 (TSP-1)
Platelet-derived endothelial cell growth factor (PD-ECGF) (thymidine phosphorylase)	Tissue inhibitors of metalloproteinases -1 and -4 (TIMP-1 and TIMP-4)
Angiopoietin-1 (ANGPT1)	Plasminogen activator inhibitor-1
Matrix Metalloproteinases 2 and 9 (MMP-2 and MMP-9)	
Lipoprotein A (LPA)	
Sphingosine-1-phosphate (S1P)	
Stromal Cell-Derived Factor (SDF-1; CXCL12)	
Heparanase	
Deoxyribose-1-Phosphate	

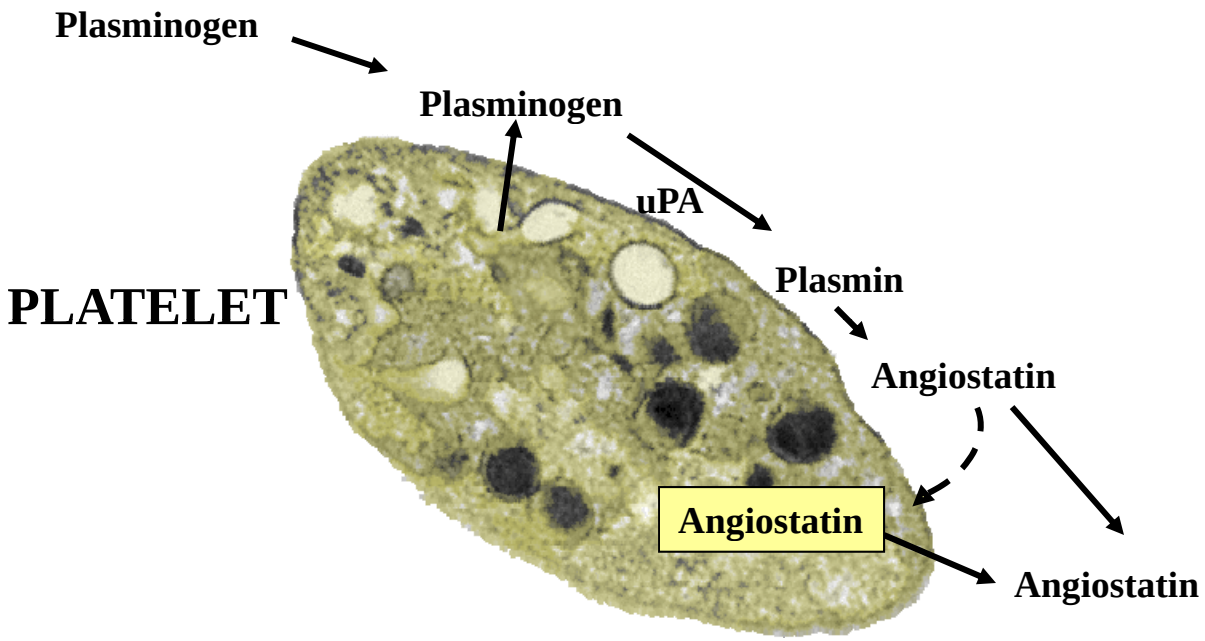
Plasminogen
Plasminogen
uPA
PLATELET
Plasmin
Angiostatin
Angiostatin
Angiostatin

Figure 1

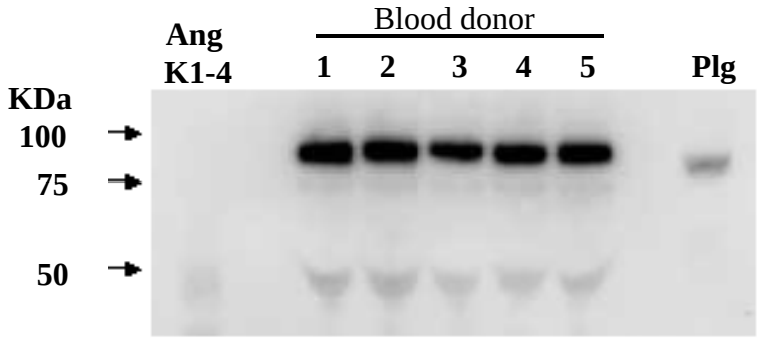
Blood donor
Ang K1-4
1
2
3
4
5
Plg
KDa
100
75
50
Human plasma (from healthy volunteers)

Figure 2