Minireview

The Hemostatic System as a Regulator of Angiogenesis*

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Angiogenesis is the process of sprouting and configuring new blood vessels from pre-existing blood vessels, whereas the hemostatic system maintains the liquid flow of blood by regulating platelet adherence and fibrin deposition. Both systems normally appear quiescent, yet both systems remain poised for repair of injury. With vessel injury, a rapid sequence of reactions must occur to occlude the vessel wall defect and prevent hemorrhage. Activated platelets link the margins of the defect and form a provisional barrier that is quickly emmeshed with polymerized fibrin. This clot structure initially requires immobilized vascular endothelial cells to anchor the clot and prevent further bleeding. Thereafter, endothelial cells at the clot margins become mobile, dismantling and invading the cross-linked fibrin structure to rebuild a new vessel wall.

Although the positive and negative regulators that control the delicate balance of platelet reactivity and fibrin deposition have been elucidated over the past four decades, analogous proteins that control endothelial cell growth and inhibition have only been discovered within the past decade. Hemostasis and angiogenesis are becoming increasingly inter-related. Proteins generated by the hemostatic system coordinate the spatial localization and temporal sequence of clot/endothelial cell stabilization followed by endothelial cell growth and repair of a damaged blood vessel. We focus here on the regulation of angiogenesis during vessel repair mediated by proteins secreted by platelets and derived as cryptic fragments from the coagulation cascade and fibrinolytic system.

Platelets Contain Regulators of Angiogenesis

At the site of vessel injury, adhered platelets secrete both positive and negative regulators of angiogenesis, mainly from internal α-granules. These positive regulators include: vascular endothelial growth factor-A (VEGF-A)1 (1), VEGF-C (2), basic fibroblast growth factor (bFGF) (3), hepatocyte growth factor (HGF) (4), angiopoietin-1,2 insulin-like growth factor-1 (IGF-1), Plasminogen activator inhibitor type 1 (PAI-1), Plasminogen activator inhibitor type 2 (PAI-2), tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), angiopoietin-1,2 angiopoietin-2, integrin αvβ3, integrin αvβ5, integrins αvβ6, αvβ8, and αvβ1, fibronectin (FN), laminin, collagen, and von Willebrand factor. These α-granule contents represent the functional armamentarium of platelets. When released following platelet secretion, these proteins secrete these proteins to regulate angiogenesis.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; PF4, platelet factor 4; AT-III, antithrombin III; TSP-1, thrombospondin; TGF-β1, transforming growth factor-β1; PAI-1, plasminogen activator inhibitor type 1; uPA, urokinase-type plasminogen activator; HMWK, high molecular weight kininogen; MMP, matrix metalloproteinase.

2 G. D. Yancopoulos, personal communication.

HGF—In contrast to the multiple pro-angiogenic activities of HGF (13, 14), alternative processing of the HGF α-chain mRNA generates anti-angiogenic HGF fragments consisting of either the first kringle domain (NK1) or the first two kringle domains (NK2) (14). These first two kringle domains contain the HGF binding site for its receptor, c-Met (14). NK1 and NK2 suppress HGF-induced endothelial cell migration and abrogate HGF-induced angiogenesis in the rat cornea (14). These observations led to recombinant construction of NK4, which contains all four kringle domains of HGF (15). HGF/NK4 is a more potent antagonist of c-Met activation by HGF (15). HGF/NK4 potently inhibits tumor growth in vivo by increasing tumor cell apoptosis without affecting the proliferation rate of tumor cells (15). A similar pattern of tumor inhibition occurs through angiogenesis inhibition (16). Taken together, the anti-tumor activity of HGF/NK4 in vivo is at least partly mediated through an anti-angiogenic activity (15). Thus, expression of NK1 or NK2 or cryptic cleavage of HGF into NK1, NK2, or NK4 could counterbalance HGF-induced angiogenesis in vivo.

Platelet Factor 4 (PF4)—Unique to platelets, PF4 binds surface heparin-like glycosaminoglycans on endothelial cells, thereby quenching the anti-thrombotic activity of antithrombin III (AT-III) and allowing a clot to form. Nearly two decades ago, PF4 was the first hemostatic protein demonstrated to be an inhibitor of angiogenesis in vivo (17, 18). One mechanism for the initial endothelial cell inhibition following platelet secretion is that PF4 blocks heparin-like glycosaminoglycans that function as critical, low affinity receptors for heparin-binding endothelial growth factors on the surface of endothelial cells (18). PF4 also directly neutralizes the heparin-binding region of VEGF-A121, endothelial mitogens that lack heparin affinity, is susceptible to PF4 inhibition (20). Moreover, an analogue of PF4 that lacks heparin affinity (rPF4-241) inhibits angiogenesis (21).

Thrombospondin (TSP-1)—TSP-1 is the most abundant constituent of platelet α-granules and participates in efficient platelet aggregation (22, 23). TSP-1 is a large (450 kDa), modular glycoprotein complexed with active transforming growth factor-β1 (TGF-β1) in α-granules and, upon release, can activate latent TGF-β1 secreted by endothelial cells (24). TSP-1 binds fibrin (25), fibronectin (26), plasminogen (27), surface heparin-like glycosaminoglycans (26), CD36 and αvβ3 integrins on activated endothelial cells (27), and αvβ3 integrins on activated platelets (27). TSP-1 may re-adjust growth factor and integrin signaling pathways between endothelial cells and the fibrin clot (27) and prevent endothelial cell motility induced by fibrin. TSP-1 stimulates endothelial cell adhesion and spreading but blocks the chemokinetic response of endothelial cells to bFGF (28).

TGF-β1—Platelet α-granules are a rich source for active TGF-β1 (29). TGF-β1 promotes the formation of quiescent cap-
illar tubules in vitro and mediates potent inhibition of endothelial cell proliferation and migration (30). TGF-β1 blocks the proliferation of endothelial cells to even supramaximal concentrations of bFGF (30). In vivo, however, TGF-β1 induces angiogenesis (30, 65) that is thought to reflect recruitment of macrophages, which secrete endothelial cell growth factors (30).

Plasminogen Activator Inhibitor Type 1 (PAI-1), α2-Antiplasmin, and α2-Macroglobulin—Regulation of plasminogen activation is critical to the sequence of stable fibrin clot formation followed by controlled fibrin digestion. PAI-1 is maintained in an active conformation in complexes with vitronectin within platelet α-granules (31). Platelet-derived PAI-1 prevents initial fibrinolysis of platelet-rich thrombi (31) but is less effective in the inhibition of the endothelial cell membrane-associated plasminogen activator (uPA) activity (37) that is generated by endothelial sprouts (33, 34) (Fig. 1). By limiting plasmin generation within the clot structure, PAI-1 can suppress angiogenesis (32, 66) (Fig. 1). By scavenging plasmin, platelet-derived and fibrin-bound α2-antiplasmin (35) and α2-macroglobulin (36) may also negatively regulate angiogenesis (37).

Negative Regulators of Angiogenesis within Coagulation Cascade

High Molecular Weight Kininogen (HMWK) Domain 5—HMWK circulates in plasma bound to prekallikrein. Contact activation of this complex can begin the coagulation cascade. Kallikrein-cleaved HMWK (Hka) and vitronectin compete for binding to the endothelial cell urokinase receptor (67). Cryptic generation of Domain 5 (Lys420–Ser513) from Hka inhibits the migration of endothelial cells to vitronectin and fibronectin, both components of the fibrin clot. Domain 5 of HMWK also inhibits endothelial cell proliferation and is anti-angiogenic on the chicken chorioallantoic membrane (38).

Fragment-1 and -2 of Prothrombin—Activated coagulation factor Xa cleaves factor II (prothrombin) to yield thrombin and a two-kringle amino-terminal domain (fragment 1–2) (39). Thrombin then cleaves fragment 1–2 of prothrombin into single-kringle fragment-1 and fragment-2. Thrombin induces angiogenesis in vivo via cleavage of the tethered ligand of the thrombin receptor on endothelial cells without the requirement for fibrin formation (40). Simultaneously, these two amino-terminal kringle domains of prothrombin are released. Fragment-1 and fragment-2 of prothrombin inhibit the proliferation of endothelial cells in vitro and angiogenesis in vivo (39). Thus, the stimulatory effects of thrombin on endothelial cells would be antagonized by kringle by-products released upon activation of prothrombin.

AT-III—In the presence of heparin, AT-III avidly inhibits the activated form of factors II (thrombin) and X in plasma. This inactivation is very inefficient when coagulation factors are bound to the anionic phospholipid surface of activated platelets and endothelial cells. AT-III thus serves an important physiologic role in limiting the extent of an evolving clot to the area of vascular injury. Thrombin and neutrophil elastase can cleave the thrombin-binding site of AT-III (41). Once generated, cleaved AT-III (anti-angiogenic AT-III) becomes a potent inhibitor of endothelial cell proliferation in vitro and angiogenesis in vivo (41). Thus, the angiogenic activity of thrombin generated at the site of clotting may be balanced not only by fragment-1 and -2 of prothrombin but also through production of anti-angiogenic AT-III.

Haptotaxis and Capillary Tube Formation Induced by Fibrin

Thrombin cleaves small peptides from the amino-terminal ends of the α and β chains of soluble fibrinogen to form insoluble fibrin monomers. Fibrin monomers self-assemble at the site of vessel injury and enmesh the adhered platelets, migration-inhibited endothelial cells, and the exposed subendothelial matrix. Along with binding of latent regulators of plasminogen activation, fibrin also displays high affinity binding for bFGF (42), delivered by platelets. This fibrin gel is covalently cross-
linked over the next several hours by thrombin-activated Factor XIII. In vitro, fibrin acts as a scatter factor on confluent endothelial cells (43), an effect that would be counterproductive during hemostasis and therefore must be initially counteracted (see “Discussion”). Fibrin mediates endothelial cell adhesion and spreading via endothelial cell αβ3 integrin binding to the RGD motifs at positions 252–254 and 572–574 of its α-chain (44). Migration into fibrin gels requires growth factor-stimulated endothelial cell uPA receptor, uPA, and resulting plasminogen activation (33, 34). Localized production of plasmin at the endothelial invasion front lowers the density of the fibrin matrix required for capillary tube formation (45). As endothelial cells migrate into and align within the more dilute and flexible fibrin gel, residues 15–42 on the β-chain of fibrin interact with vascular endothelial cadherin on endothelial cells and facilitate capillary morphogenesis (46). Thus, fibrin plays a central role in the sequential events of vascular repair. First, fibrin tightly secures the platelet plug over immobilized endothelial cells to prevent hemorrhage. Second, fibrin serves as a sustained release reservoir for endothelial growth factors (42) and fibrinolytic enzymes (47). Third, as the clot is dismantled, partially digested fibrin provides solid-state guidance of endothelial cell migration (44, 48) and capillary tube formation (46).

A Negative Regulator of Angiogenesis within the Fibrinolytic System

Plasminogen is bound to the clot structure and is initially prevented from activation (31, 49). Angiotatin, kringle 1–4 of plasminogen, is a circulating inhibitor of angiogenesis originally discovered by its ability to prevent the growth of cancer metastases (50). Angiotatin potently and specifically inhibits endothelial cell proliferation in vitro and angiogenesis in vivo (50, 51). Further, portions of all five kringle domains of plasminogen/plasmin possess anti-angiogenic activity (52, 53). Angiotatin binds to the α/β-subunits of UTP synthase on the surface of endothelial cells, potentially inducing H+ cyttoplasmic influx into endothelial cells and cytolysis (54). Several mechanisms have been demonstrated to generate biologically active angiotatin. These include: (i) cleavage by active matrix metalloproteinase (MMP) -2 (55), MMP-3 (56), MMP-7 (57), and MMP-9 (57); (ii) cleavage by a tumor cell-derived plasmin/thiolreductase (58, 59); and (iii) cleavage of plasminogen on the surface of macrophages by granulocyte-macrophage colony-stimulating factor-induced metalloelastase (MMP-12) (60). Angiotatin also governs the rate of plasminogen activation through non-competitive inhibition of tissue-type plasminogen activator (61). Thus, generation of angiotatin may regulate the speed of endothelial cell migration and proliferation into the clot both directly and through feedback inhibition of plasminogen activation.

Discussion

During the first days as the nascent clot bridges and stabilizes the vessel defect, any initiation of angiogenesis directed by platelet-derived positive regulators, thrombin, and fibrin must be counteracted. Following clot stabilization, angiogenesis must be tightly regulated to avoid re-bleeding. This regulation is achieved through proteins secreted by platelets and cryptic fragments generated from hemostatic proteins involved in coagulation and fibrinolysis. Although the timing of release of these cryptic fragments is unknown, we can speculate that they may operate when known proteolytic activities develop (5, 34, 37, 62). Platelet secretion is triggered within the first few minutes of hemostasis and results in deposition of both positive and negative regulators of angiogenesis. Platelet-derived PF4, TSP-1, and TGF-β1 may counteract immediate endothelial cell migration and proliferation resulting from platelet-derived positive regulators of angiogenesis and fibrin. In this early context, these positive regulators of angiogenesis derived from platelets may function as anti-apoptotic factors (63, 64). Cleavage of prothrombin to thrombin and fragments 1 and 2 occurs concomitantly with platelet secretion. Further, some of the thrombin that is generated may cleave local AT-III. Thus, fragments 1 and 2 of prothrombin and anti-angiogenic AT-III could also antagonize the initial pro-angiogenic stimulus from platelets, thrombin, and fibrin. PAI-1, α2-antiplasmin, and α2-macroglobulin initially prevent plasmin activity. Thereafter, pericellular fibrinolysis and angiogenesis are initiated by endothelial cell expression of the uPA receptor (34) and uPA (34) and also membrane type-1 MMP (5). Focal generation of angiotatin could then occur via the mechanisms discussed and would regulate both the speed of endothelial repair and rate of plasmin production. Cryptic fragments of HMWK and HGF may also be generated during fibrinolysis. Thus, angiotatin, HMWK domain 5, and HGF/NK1, NK2, or NK4 may limit excessive angiogenesis induced by the unopposed activity of platelet-derived angiogenic growth factors and fibrin during fibrinolysis. Platelet secretory proteins and cryptic fragments generated during clotting and fibrinolysis may sequentially induce endothelial cell immobilization during hemostasis and control the rate of angiogenesis during vessel repair.

The capacity of the hemostatic system to store proteins that regulate angiogenesis provides a new conceptual framework to understand how angiogenesis is coordinated by and with hemostasis during vessel repair.

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