Thrombotic Regulation From the Endothelial Cell Perspectives

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Thrombosis, the localized clotting of blood that affects arterial or venous circulation, is one of the leading causes of death worldwide.[1] Currently used antithrombotic drugs have increased bleeding risk.[2,3] Exploring new antithrombotic strategies that preserve physiological hemostasis is under intensive investigation. Classical triad of causative factors leading to thrombosis includes alterations in blood content, alterations in blood flow, and alterations in the vessel wall. Endothelium, the inner most single layer of cells lining the blood vessels, provides a surface for thrombosis formation and critically regulates blood fluidity and homeostasis. As barrier, endothelium separates blood clotting factors from exposure to subendothelial prothrombotic extracellular matrix components. Endothelium also secretes or expresses vasoactive factors that modulate platelet reactivity, coagulation, fibrinolysis, and vascular contractility, all of which contribute to thrombotic formation. Such factors include nitric oxide, prostacyclin, Von Willebrand factor (VWF), thrombomodulin, endothelin, etc. Accumulating evidences show that endothelial cells (ECs) play a pivotal role in modulating thrombosis, highlighting ECs as a potential target for thrombosis control. In this Recent Highlights, we reviewed recent publications in *Arteriosclerosis, Thrombosis, and Vascular Biology* and other leading journals that are focused on the mechanisms of endothelial regulation of thrombosis and discussed their merits in therapeutic discovery.

Arterial thrombosis is commonly initiated by vascular endothelial erosions and ruptures of an atherosclerotic plaque[3,4] while venous thrombosis mainly stems from blood stasis.[5] Despite these differences, platelet adhesion/activation, and fibrin deposition as the result of coagulation constitute the fundamental processes of thrombus formation.[6,7] Platelet activation occurs when they interact with activated ECs (with increased VWF release and selectin expression) via glycoprotein Ib-V-IX complex binding to VWF, or when they expose to subendothelial extracellular matrix components, such as collagen (via glycoprotein VI receptor), in the cases of endothelial injury or plaques rupture.[1,8] Coagulation cascade is triggered by coagulation factor VII binding to tissue factor (TF; extrinsic pathway) or by contact system activation via factor XII (FXII; intrinsic pathway), followed by a common pathway that leads to fibrin formation through intricate enzymatic actions of different coagulation factors (Figure).[1] Recent findings on the mechanisms of endothelial regulation of thrombosis are highlighted in this review, with relevant factor as subtitle.

Von Willebrand Factor

VWF is a large multidomain adhesive glycoprotein.[9,10] VWF binds to platelet glycoprotein Ibα, αIIbβ3 and subendothelial collagens, which activates platelets and initiates platelet aggregation.[9] As an important carrier of coagulation factor VIII, VWF also contributes to blood coagulation.[11] VWF is synthesized by ECs and megakaryocytes and is stored as ultra large VWF multimers or high molecular weight multimers in the endothelial Weibel–Palade bodies or the platelet α-granules, respectively.[12] Plasma VWF was thought to be derived from the secretion of ECs and platelets and could be enhanced by secretagogues.[12] Although numerous studies have revealed the roles of VWF on hemostasis and coagulation, the differences between the endothelial VWF and the platelet VWF in thrombotic and thrombosis-related diseases are unclear. Dhanesha et al[13] examined the roles of endothelial and platelet VWF in thrombosis in vivo. Using bone marrow transplantation, they generated the platelet (Plt)-VWF mice that express VWF in hemocytes but not in ECs and the EC-VWF mice in which VWF were only expressed in ECs. The results showed that Plt-VWF mice but not EC-VWF mice exhibited defective arterial thrombotic response to $FeCl_3$ treatment, indicating a critical role of endothelial VWF in thrombosis. Furthermore, they analyzed the contributions of Plt-VWF in thrombosis in the EC-VWF defective mice and found a minor involvement of Plt-VWF in thrombus formation. Thus, endothelial VWF but not platelet VWF critically promotes thrombosis formation, raising a possibility of specific suppressing EC-VWF for antithrombosis benefit. In line with this, EC-VWF, but not Plt-VWF, contributes to VWF-dependent atherosclerosis by promoting platelet adhesion and vascular inflammation.[14] Endothelial VWF is also essential for hemostasis in tail-transection bleeding assay.[13]

Tissue Factor

TF is the activator of extrinsic coagulation system and triggers both arterial and venous thrombosis.[15] It binds to and activates coagulation factor VII, and TF-VIIa complex then activates factors IX, X, and thrombin, resulting in fibrin formation. TF is primarily expressed in the perivascular cells and epithelial cells. Platelets and neutrophils also express TF. In general, TF is not highly expressed in ECs.[15] However, the expression of

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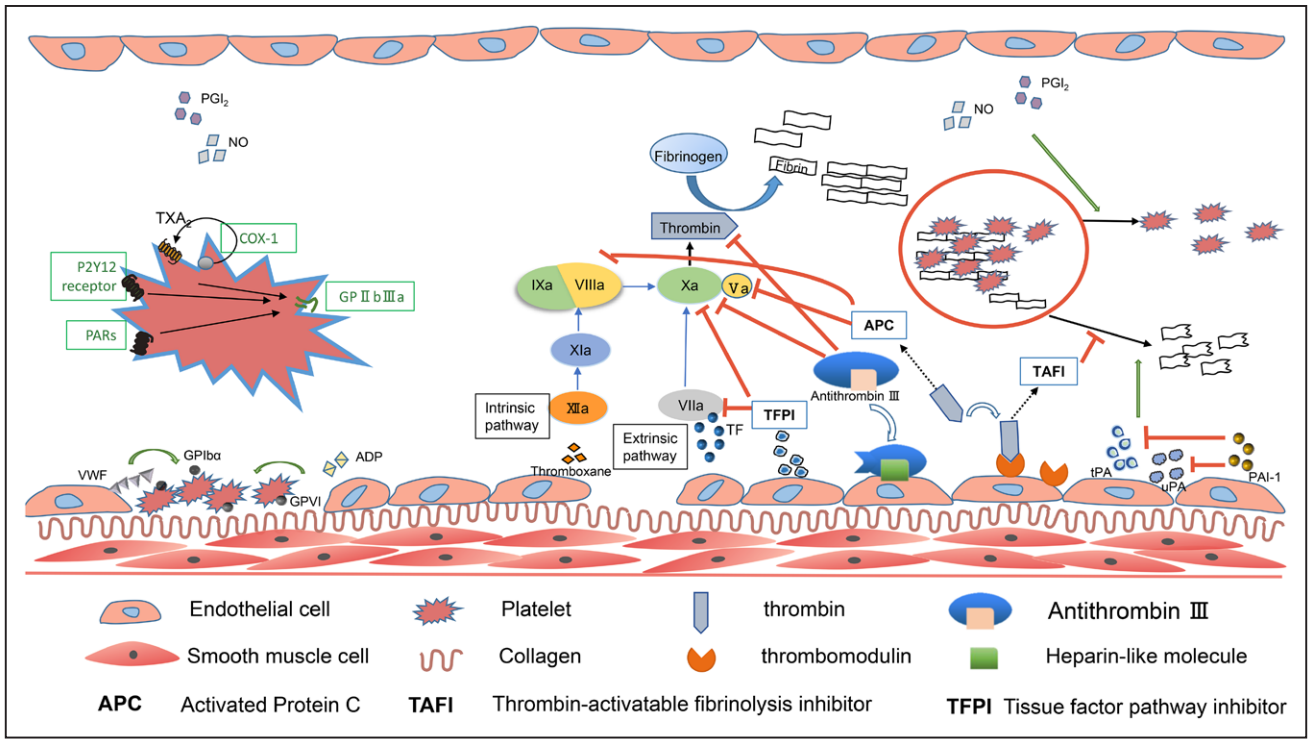

Figure. Endothelial regulation of thrombosis. Activation of platelet and coagulation, together with thrombolysis, converges at the endothelium and is subjected to endothelial regulation. APC indicates activated protein C; GP, glycoprotein; NO, nitric oxide; PAI, plasminogen activator inhibitor; TF, tissue factor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; and VWF, Von Willebrand factor.

endothelial TF may be enhanced in pathophysiological conditions,[16,17] and ECs were found to be able to secret microparticles rich in TF.[18] Indeed, endothelium-derived TF functionally participates in thrombosis.[19–24] In this regard, Witkowski et al[25] identified that circulating miR-126, which was primarily produced by ECs,[26] attenuated thrombosis via decreasing the expression of both mRNA and protein of TF. Their study showed that levels of plasma miR-126 were negatively correlated with concentrations and activities of plasma TF in diabetic patients. Furthermore, antidiabetic treatment increased the levels of miR-126 while decreased the levels of plasma TF, indicating a negative modulation of plasma TF by miR-126 in clinical conditions. Finally, by treatment of miR-126 in human microvascular ECs, they revealed that miR-126 indeed negatively regulated TF expression via interacting with the F3-3′-untranslated region of the TF mRNA. This study uncovers a novel mechanism of endothelial TF expression via miR-126 and suggests potential of modulating endothelial TF to prevent thromboembolic events in patients with metabolic diseases or at risk.

However, TF pathway inhibitor (TFPI) is the major endogenous anticoagulant protein. It inhibits TF activation by directly inhibiting factor Xa (FXa), and Fxa dependently prevents factor VIIa/TF activities.[27] Alternatively spliced isoforms of TFPI are differentially expressed by ECs and platelets and plasma. The TFPIβ isoform localizes to the endothelium surface where it is a potent inhibitor of TF–VIIa complex that initiates blood coagulation. In procoagulating conditions, like atherosclerotic plaques, TFPI colocalizes with TF, indicating an active role of TFPI in attenuating TF activity.[28] Alan Mast[29] recently reviewed the anticoagulation activities of TFPI and its clinical relevance. TFPI may be a promising target for treating thrombosis/bleeding disorders.[28–30] TFPI is believed to be secreted primarily by ECs,[27,31] thus could be an important messenger of ECs in modulating TF activities and thrombosis.

Thrombomodulin

Thrombomodulin is a vasoactive factor highly expressed on the surface of ECs.[32] Thrombomodulin exerts a potent anticoagulation effect by binding to thrombin, which directly decrease the levels of circulating thrombin, and it also inactivates factors Va and VIIIa by potentiating the generation of activated protein C.[33] Thrombomodulin protects ECs and vasculature by depressing inflammatory injuries.[34] Elevation of circulating thrombomodulin via directly delivering recombinant thrombomodulin attenuated thrombotic inflammation in experimental animals and improved clinical outcomes in patients with sepsis and suspected disseminated intravascular coagulation.[35,36] Another study suggests that enhanced endothelial expression of thrombomodulin might be thrombosis protective.[37] Thus, delineation of the regulatory mechanism of endothelial expression of thrombomodulin seems to be therapeutically relevant. Yang et al[38] identified Nor1 and Nur77 (both belongs to nuclear receptor 4A family) as novel regulators of thrombomodulin expression in ECs. Increased expression of Nor1 and Nur77 elevated endothelial expression of thrombomodulin, through enhancing transcription and post-transcriptional mRNA stability, respectively. Furthermore, enhanced expression of Nur77 and Nor1 protects mice from arterial thrombus formation. Although these nuclear receptors

may have multiple functions in different cell population, this study highlights a central role of ECs in regulation of thrombosis via producing thrombomodulin, raising a possibility to prevent thrombosis by targeting endothelial nuclear receptors.

Protein C

Protein C, also known as blood coagulation factor XIV, is a zymogen. Activated protein C regulates anticoagulation, inflammation, cell death, and maintains vascular permeability.[39,40] These functions are primarily mediated by proteolytically inactivating factor Va and factor VIIIa. Patients with deficiencies in protein C experience increased risk of thrombosis. The protein C zymogen is activated when it binds to thrombin, and protein C's activation is promoted by the presence of thrombomodulin and endothelial protein C receptors. As such, its activation may be considered as by thrombin–thrombomodulin (or even thrombin–thrombomodulin–endothelial protein C receptors) complex. On the endothelium, activated protein C performs a major role in regulating blood clotting, inflammation, and cell death. Despite commonly used murine model of atherosclerosis is resistant to thrombus formation, silencing of protein C in apolipoprotein E–deficient mice with small interfering RNA allows occurrence of spontaneous atherothrombosis at low incidence.[41] It is unclear whether this prothrombotic effect is only attributed to the anticoagulation of activated protein C[42] or also to its cytoprotective effects in anti-inflammation, antiapoptosis, and stabilization of endothelial barriers.[43]

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1),[44] also known as endothelial PAI, is a serine protease inhibitor (serpin). It functions as the principal inhibitor of tissue- or urokinase-type plasminogen activator, and hence fibrinolysis, with additional nonfibrinolytic function recently discovered.[45–47] PAI-1 is mainly produced by the endothelium but is also secreted by other tissue types, such as adipose tissue. Elevated PAI-1 is a risk factor for thrombosis and atherosclerosis.[48] Transgenic mice overexpressing PAI-1 developed age-dependent coronary arterial thromobosis. Recent studies reviewed by Vaughan et al[46] suggest PAI-1 as a mediator of cellular senescence. Normalization/inhibition of elevated PAI-1 might be a new strategy to control age-associated pathologies, including thrombosis, arteriosclerosis, obesity, diabetes mellitus, among which endothelial dysfunction/senescence represents a common pathology.

Reactive Oxygen Species

Reactive oxygen species (ROS) is known to regulate thrombosis.[49–51] The general sources of ROS in vascular system include dihydronicotinamide adenine dinuclectide phosphate oxidases, xanthine oxidase, uncoupled endothelial nitric oxide synthase, and leakage of activated oxygen from mitochondria during oxidative respiration.[50] ROS regulates EC biology.[37,52–55] However, evidence on thrombosis regulation by EC-derived ROS is limited. Antioxidative treatment inhibits the release of thrombogenic TF from irradiation- and cytokine-treated ECs, indicating a significant role of endothelial ROS in thrombotic regulation.[56] Furthermore, endothelial-specific deletion of Txnrd2, an enzyme negatively regulates the levels of mitochondrial ROS, robustly enhances fibrin deposition.[57] In line with this study, Li et al[58] showed that mitochondrial ROS was responsible for lysophosphatidylcholine-induced EC activation. Particularly, ROS modulated by Txnrd2 in the endothelial compartment promoted thrombus formation.[57] Although ROS can be produced by various types of cells that may involve in thrombosis,[42] these studies shed new light on a key role of endothelial ROS in regulating thrombosis. This mechanism is in concert with the role of platelet ROS in thrombus formation: platelet-specific deficiency of class III phosphoinositide 3-kinase (also known as vacuolar protein sorting 34) attenuates thrombosis via influencing nicotinamide adenine dinuclectide phosphate oxidases assembly.[59] Given the complexity of ROS signals, how to therapeutically modulate the levels of endothelial ROS to curtail thrombosis warrants further study. Interestingly, in a randomized, placebo controlled clinical trial in patients with antiphospholipid syndrome, treatment with ubiquinol, the reduced equivalent of coenzyme Q10, improved endothelial function and reduced thrombotic risk markers, likely through reduced EC inflammation related to oxidative stress.[60]

Endothelium–Blood Cell Interaction

Adhesion of leukocytes to ECs contributes to thrombosis, especially venous thrombus formation.[61–63] Growth arrest–specific 6 promotes venous thrombosis via enhancing the interactions of ($CCR2^{hi}CX3CR1^{lo}$) monocytes with ECs.[64] The interactions rely on enhanced endothelial expression of CCL2 (C-C chemokine ligand 2), a chemokine ligand, highlighting a key role of ECs in modulating thrombosis via leukocyte chemotaxis. Intriguingly, VWF mediates erythrocyte–endothelium interaction, and this interaction might contribute to venous thrombus formation.[65] Preventing interactions of ECs with blood cells could be a new strategy to reduce thrombosis, especially venous thrombosis.

Endothelium Integrity

Besides secretion or expression of above mentioned mediators of thrombosis, endothelial integrity per se influences thrombotic response. To reduce bleeding risk associated with systemic delivery of thrombin inhibitors, Palekar et al[66] administered plaque-localizing nanoparticles carrying a potent thrombin inhibitor in atherosclerotic mice. This approach restored endothelial barrier and attenuated atherogenic inflammation and thrombotic risk.[66] Thus, thrombin may indirectly promotes thrombosis via impairing endothelial barrier function beyond its known coagulating activity. The efficacy of clinical use of drug-elute stent is limited by in-stent thrombosis, which is attributed to impaired endothelium. In a rabbit model of atherosclerosis, peroxisome proliferator–activated receptor-δ ligand-coated stent promotes vessel re-endothelialization and prevents thrombocyte activation. This is consistent with a mechanism by which improving endothelial repair via peroxisome proliferator–activated receptor-δ activation limits thrombotic risk.[67] The ameliorated prothrombotic status after reduced equivalent of coenzyme Q10 treatment might also reflect an improved functional integrity of the endothelium in

patients with antiphospholipid syndrome.[60] Therefore, maintaining structural and functional integrity of EC is a key determinant of thrombotic risk and has therapeutic value.

Contact Activation

Contact activation via coagulation FXII to form its active form (FXIIa) initiates the intrinsic coagulation cascade. Contact activation arises its name from FXII's unique mechanism of activation that is induced by binding (contact) to negatively charged surfaces. Various substances have the capacity to trigger FXII contact activation in vivo, including mast cell–derived heparin, collagen, and polyphosphate. The role of endothelium in contact activation is unclear. Preclinical evidence supports that pharmacological inhibition of FXII/FXIIa may be a promising therapeutic anticoagulation treatment strategy with less bleeding risk.[68] However, this promise awaits confirmation in clinical trials.

Heparin is a naturally occurring anticoagulant that is usually stored within the secretory granules of mast cells and released only into the vasculature at sites of tissue injury. Heparin binds to antithrombin III, causing a conformational change that results in its activation. The activated antithrombin III then inactivates thrombin, Fxa, and other proteases. How to determine the functions of heparin on live ECs remains challenging. Dimitrievska et al[69] developed a method that can quantify functional heparin weight on live endothelialized surface and their anticoagulant capacity to inactivate FXa and thrombin. Using this novel approach, they reported a striking difference (≈10-folds) in heparin weight on native aorta and cultured human umbilical vein ECs, which is consistent with the lower anticoagulation capacity of human umbilical vein ECs in inactivating both FXa and thrombin relative to native aortas.[69] This method can be valuable for future study of endothelial anticoagulation activity and its molecular regulation in vitro.

Summary

Endothelium maintains blood fluidity through delicate regulation of platelet reactivity, coagulation, and thrombolysis, by synthesizing and responding to vasoactive molecules. Loss of endothelial normal function or structural integrity results in acute thrombosis, or chronic vascular changes that predispose to thrombosis among a variety of diseases, such as atherosclerosis, restenosis, diabetes mellitus, obesity, etc.[70–73] Understanding the mechanism by which endothelium regulates thrombosis not only provides insights into thrombosis mechanism and pathology of relevant diseases but also fuel the therapeutic endeavor for discovering effective antithrombotic drugs with minimal or no bleeding risk, which constitute a substantial unmet medical need.

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Disclosures

None.

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