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Molecules in focus

The role of bone marrow-derived cells in venous thromboembolism

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ABSTRACT

Venous thrombosis is a life-threatening condition with high morbidity and mortality. Abnormal functioning of different cells in the blood is an integral part of its pathogenesis. In this review, we describe the contribution of bone marrow-derived cells to the development of this debilitating disease. We present both epidemiological and clinical data demonstrating involvement of various cell types in venous thrombosis, and discuss potential mechanisms underlying these effects. Modern concepts including recently discovered new paradigms in thrombosis, such as neutrophil extracellular traps, mast cells, and polyphosphate, are summarized.

1. Introduction

Formation of blood clots in deep veins, known as deep vein thrombosis (DVT) is a severe debilitating disease. In addition to the local symptoms impairing the quality of life (such as redness, swelling, pain and gait defect) (Budnik and Brill, 2018), detached clots can travel to the lungs and obstruct the pulmonary artery, resulting in respiratory insufficiency. This complication, known as pulmonary embolism (PE), requires immediate medical attention and can lead to death. DVT and PE are designated together as venous thromboembolism (VTE). DVT develops in ~900,000 Americans annually (Heit, 2008). The estimated total burden in six European countries was ~470,000 cases of DVT, ~300,000 cases of PE, and ~370,000 VTE-related deaths (Cohen et al., 2007).

Factors predisposing to DVT include such conditions as hereditary hypercoagulability, cancer, pregnancy, or trauma (Goldhaber, 2010). However, in many cases, idiopathic DVT is driven by prolonged immobility, for example, after major surgery or long-haul flights (Goldhaber, 2010). Pathophysiologically this corresponds to blood flow stagnancy, which triggers thrombus formation (Bovill and van der Vliet, 2011).

Although a blood clot forms as a result of fibrin generation in the coagulation cascade, recent findings suggest that DVT starts from a cascade of events resembling local sterile inflammation. These events include local activation of the endothelium (most likely because of local hypoxia due to the lack of blood flow) followed by the recruitment of leukocytes and platelets (Budnik and Brill, 2018). In experimental models of DVT, such as inferior vena cava (IVC) stenosis, these processes have been shown to be pivotal for thrombosis initiation. In this review, we will discuss roles of different bone marrow-derived cell types in DVT and potential of their targeting for thrombosis prevention.

2. Platelets

Although venous thrombi are red, i.e., consist predominantly of red blood cells (RBCs) and fibrin, platelets are incorporated in human venous clots in the form of lines of Zahn (Esmon, 2009). A retrospective study of all in-hospital patients demonstrated that platelet count higher than $350 \times 10^3/\mu l$ is typical for venous thrombosis complications (Zakai et al., 2004). High platelet count is associated with increased risk of VTE in cancer patients (Simanek et al., 2010). Polymorphism of certain platelet receptors, such as alpha2A adrenergic receptor (ADRA2A), platelet endothelial aggregation receptor 1 (PEAR1), or GPVI are associated with the risk of DVT (Bezemer et al., 2008; Montoro-Garcia et al., 2016; Sokol et al., 2018), although the mechanism for this remains unclear. Interestingly, mean platelet volume has also been reported to be an independent predictor of venous thrombosis (Braekkan et al., 2010b; Cil et al., 2012). In an experimental model of DVT, platelet depletion protected mice from thrombosis (von Bruhl et al., 2012). Moreover, inhibition or genetic ablation of P2Y1, a receptor for platelet activation with ADP, reduced DVT in animal models (Bird et al., 2012; Lenain et al., 2003). In patients, some clinical studies demonstrated usefulness

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of aspirin in prevention of primary DVT and PE as well as recurrent VTE (Becattini et al., 2012; Pulmonary Embolism Prevention (PEP) Trial Collaborative Group, 2000). Aspirin inhibits platelets by suppressing cyclooxygenase-1 (COX-1)-dependent synthesis of thromboxane A2, a potent platelet agonist (Undas et al., 2007). In addition, aspirin stimulates production of strong platelet inhibitor, nitric oxide (Chakraborty et al., 2003), decrease platelet life span (Nayak et al., 2014) and reduce α-granule release (Parker and Gralnick, 1989). All these effects may be implicated in aspirin-mediated DVT prevention.

Platelet recruitment to the venous wall in the setting of stagnant flow is mediated by interaction between GPIbα on their surface and von Willebrand Factor (VWF) on the endothelium (Brill et al., 2011). Platelets can be involved in DVT through several mechanisms. First, platelets provide a surface to the clotting factor assembly via expression of phosphatidyl serine (Reddy and Rand, 2020). Platelet and endothelial microparticles carrying tissue factor, one of the major procoagulant proteins, are elevated in recurrent DVT (Ye et al., 2012). Also, platelets participate in recruitment of neutrophils that support thrombosis by releasing Neutrophil Extracellular Traps (NETs) and other routes, which will be discussed below. One of the mechanisms of platelet-dependent neutrophil recruitment includes direct interaction of choline transporter-like protein 2 (CTL2, encoded by the *Slc44a2* gene) with activated αIIbβ3 on platelets (Fig. 1). This interaction not only recruits neutrophils but also stimulates NETosis, and mice deficient for the *Slc44a2* gene have reduced venous thrombosis specifically in the flow restriction model (Constantinescu-Bercu et al., 2020; Tilburg et al., 2020).

Platelets support DVT also by releasing high-mobility group box 1 protein (HMGB1), a typical member of the death-associated molecular pattern (DAMP) family. Platelet-derived HMGB1 attracts both monocytes and neutrophils and stimulates the release of NETs and cell-free DNA. Cell-free DNA exposes additional portions of HMGB1 and activated leukocytes mediate HMGB1 oxidation increasing its prothrombotic properties and supporting platelet aggregation (Dyer et al., 2018; Stark et al., 2016). Thus, a bi-directional positive feedback is formed between pro-thrombotic and pro-inflammatory pathways upregulating venous thrombosis.

Furthermore, procoagulant platelets, a subset characterized by phosphatidylserine (PS) exposure, P-selectin expression, binding to factor Xa, ballooning and the ability to promote thrombin generation, have recently been the focus of studies looking at their role in hemostasis and thrombosis (Agbani and Poole, 2017). Interestingly, increased levels of procoagulant platelets have been reported not only in thrombotic disorders (Pasalic et al., 2018) but also in diseases with increased risk of thrombosis (Vulliamy et al., 2019; Zhao et al., 2016).

Whilst the role of procoagulant platelets in venous thrombosis has not yet been described, preclinical studies demonstrated reduced *in vivo* thrombus formation following $FeCl_3$ injury to carotid arteries, when procoagulant platelets were targeted (Agbani et al., 2015, 2018), potentially establishing a novel approach to antithrombotic therapies.

3. Neutrophils

The recruitment of neutrophils to the venous wall is an important step for DVT initiation. When stimulated, neutrophils can release NETs, a complex of chromatin fibers, histones, and enzymes, which have been shown to be involved in the development of thrombosis, and free DNA may serve as a biomarker of DVT in patients (Fig. 1) (Diaz et al., 2013; Fuchs et al., 2010; van Montfoort et al., 2013). NETs are present in experimental venous thrombi (Brill et al., 2012; Fuchs et al., 2010), and *in vivo* targeting of NETs is protective in the IVC stenosis model in mice (Brill et al., 2012), although DNase I does not affect venous thrombosis in the complete stasis model (El-Sayed et al., 2016). In these models, blood flow stagnancy, a factor driving DVT in humans, is modelled by application of partial (stenosis) or complete (stasis) flow restriction to the IVC.

Several proteins from neutrophil granules are exposed on NET strings; amongst these, neutrophil elastase promotes thrombosis by degrading the anticoagulant protein tissue factor pathway inhibitor (TFPI) (Massberg et al., 2010). Activation of peptidylarginine deiminase 4 (PAD4), which citrullinates histones and promotes chromatin decondensation, is an important step in NETosis (Li et al., 2010). Similarly to DNase-treated mice, PAD4-deficient animals are protected from thrombosis in the IVC stenosis model (Martinod et al., 2013).

Whilst in the IVC stasis model neutrophil depletion showed no effect in thrombus size (El-Sayed et al., 2016), depletion of the neutrophil population in the murine IVC stenosis model inhibits formation of venous thrombi (von Bruhl et al., 2012). Furthermore, in nude mice bearing human pancreatic tumors, a type of cancer associated with a particularly high incidence of venous thromboembolism, neutrophil depletion or destruction of NETs by DNase I administration reduces thrombus size (Hisada et al., 2020).

4. Monocytes

Along with neutrophils, monocytes are the most abundant leukocyte subset recruited to the vessel wall during DVT initiation. Monocytes are the main source of tissue factor (TF) in venous thrombi (von Bruhl et al.,

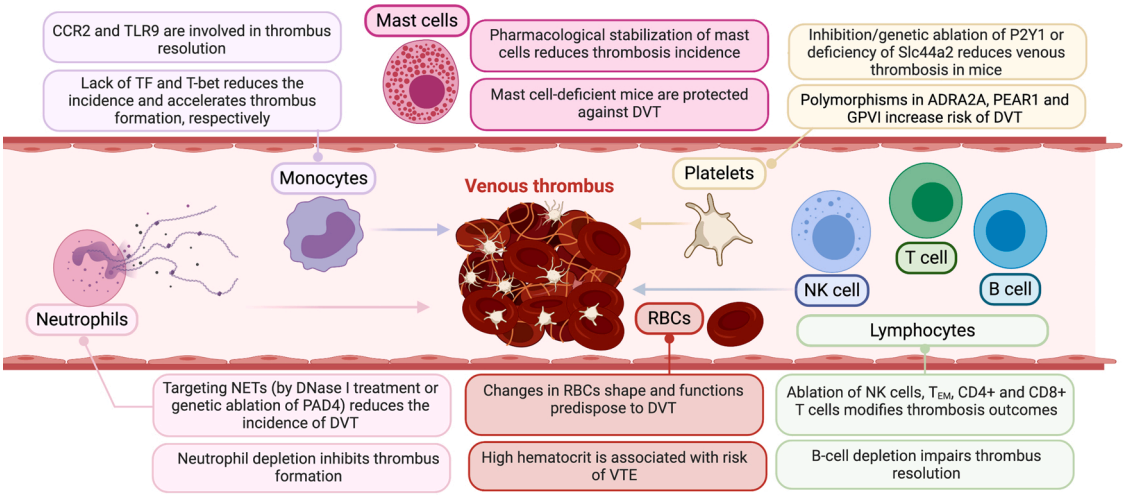

Fig. 1. Selected pathways of bone marrow-derived cell involvement in DVT. Schematic representation of routes through which cells of the innate and adaptive immune systems as well as platelets and RBCs are implicated in venous thrombosis.

2012). TF is the major initiator of coagulation and fibrin formation, and its depletion from myeloid leukocytes reduces the incidence of venous thrombosis in mice undergoing IVC stenosis (von Bruhl et al., 2012). The interaction of TF-producing myeloid cells with platelets is thought to create a procoagulant environment contributing to DVT formation (Stark et al., 2016; von Bruhl et al., 2012). However, in the stasis model of DVT, TF driving thrombus formation seems to be derived from vessel wall rather than from recruited leukocytes (Day et al., 2005).

As well as their role in DVT initiation, monocytes have also been implicated as major players in thrombus resolution, mediated, in part, by the monocyte chemoattractant protein-1 (MCP1)/CC-chemokine receptor 2 (CCR2) axis (Fig. 1) (Ali et al., 2006). In the stasis model, $CCR2^{-/-}$ mice demonstrated decreased monocyte influx, larger thrombi, at both early and later time points, as well as a higher thrombus collagen burden (Henke et al., 2006). Similarly, the absence of toll-like receptor (TLR) 9, which recognizes both foreign and host DNA, reduced the numbers of CCR2+ monocytes/macrophages in venous thrombi, and led to larger venous thrombi at days 8 and 21 after DVT induction (Dewyer et al., 2015). Moreover, $TLR9^{-/-}$ animals exhibited increased markers of NETosis, necrosis and apoptosis in the stasis model of DVT (El-Sayed et al., 2016). Genetic deficiency of CXCR2, another mediator of granulocyte chemotaxis, or treatment of experimental animals with an anti-CXCR2 antibody, led to delayed DVT resolution (Henke et al., 2004).

Monocyte phenotype may be a critical factor in determining the role of these cells in the balance of thrombus formation and resolution: the transcription factor T-bet mediates monocyte production and secretion of IL-12, a cytokine linked to interferon-γ (IFN-γ) production in the context of DVT (Luther et al., 2016). T-bet-deficient mice demonstrated reduced monocyte-derived IL-12 secretion and accelerated thrombus resolution in the IVC stenosis model (Schonfelder et al., 2018).

5. Lymphocytes

The focus of research in the field of venous thromboembolism, has principally been on the complex interplay between cells of the innate immune system and the endothelial cells at the site of pathology. A smaller amount of literature examines the role of adaptive immune cells in venous thrombosis.

Natural killer (NK) cells are key effector lymphocytes demonstrating properties of both innate and adaptive immune systems (Vivier et al., 2011). Recently, it has been reported that specific ablation of NK cells protects mice from DVT in the setting of flow restriction, which implies that NK cells are implicated in venous thrombosis (Bertin et al., 2019). Interestingly, the release of NETs was also inhibited following NK cell depletion. A set of further experiments revealed that NK cells facilitated NET-dependent DVT through production of IFN-γ. However, whether NK cells are the unique source of IFN-γ in the context of DVT remains to be verified (Becker and Reinhardt, 2019).

In another study, the decreased expression of several inhibitory (KLRB1, KLRD1, KLRF1, KLRG1) and activating receptors (KLRC1, KLRC3, KLRK1 and NCR1) on NK cells was demonstrated in patients with PE (Zhang et al., 2015). The numbers of a subtype of NK cells were also reduced in a part of patients. Similar findings were reported also in another publication (Duan et al., 2012). This implies that NK cells might play a role also specifically in PE. Importantly, targeting NK cells is currently emerging as an approach to treat cancer (Ghaemdoust et al., 2019), which can by itself be a risk factor for VTE, and therefore further investigation of the role of NK cells in venous thrombosis is of great importance.

The role of B cells in venous thrombosis is less clear, although B-cell depletion resulted in impaired thrombus resolution in the murine stasis model of DVT (Frey et al., 2012).

The role of effector memory T cells (T_{EM}) have also been investigated by using antibodies to selectively deplete the T_{EM} population prior to thrombosis induction in the murine stenosis model. This study reported significantly reduced recruitment of neutrophils and monocytes to the vessel wall and accelerated thrombus resolution (Luther et al., 2016).

In fact, both CD4+ and CD8 + T cells have been implicated in thrombus resolution, perhaps mediated through interaction with cells of the innate immune system. Depletion of T cells by an antibody, was associated with reduced numbers of infiltrating macrophages, reduced levels of the fibrinolytic marker urokinase plasminogen activator (uPA), decreased activity of metalloproteinase-9 (MMP-9), and an overall impairment of thrombus resolution (Mukhopadhyay et al., 2020).

In patients with idiopathic DVT, CX3CR1-expressing platelet-bound CD8+ lymphocytes were significantly increased and are proposed as a prognostic marker for adverse cardiovascular events (Furio et al., 2018).

6. Red blood cells

There is growing evidence of RBCs involvement in the pathogenesis of DVT. High hematocrit is associated with risk of VTE (Braekkan et al., 2010a; Eischer et al., 2012). Patients with anemia, administered with drugs promoting erythropoiesis, have increased chances to develop VTE, which is associated with elevated levels of hemoglobin (Dicato, 2008). Individuals receiving blood transfusion post-surgery had also higher risk of DVT (Gangireddy et al., 2007). Alteration of RBCs shape and functions, such as observed in sickle cell disease, predisposes to DVT and leads to increased mortality (Brunson et al., 2017).

Mechanisms of the prothrombotic effect of RBCs may include several aspects. RBCs retention in venous thrombi is mediated by plasma Factor XIII through cross-linking of fibrin α-chain (Byrnes et al., 2015; Kattula et al., 2018). The number and volume of RBCs are a major factor defining blood viscosity (Chien et al., 1975). The effect of hematocrit on blood viscosity is especially strong at low/venous shear rates (Nader et al., 2019). In accordance with the Poiseuille's Law, increased blood viscosity elevates the flow resistance and shear stress at the vessel wall. This may activate endothelial cells leading to expression of adhesion receptors and recruitment of leukocytes and platelets, which is a critical step for DVT initiation (Brill et al., 2011). RBCs push platelets to the vessel wall (a phenomenon known as platelet margination) (Czaja et al., 2020), which increases the probability of platelet-endothelial interaction. Platelet adherence to the vessel wall increases with hematocrit suggesting that RBCs directly stimulate this process (Turitto and Weiss, 1980). Elevated wall shear stress induces synthesis of nitric oxide thus promoting vessel dilation and decrease in flow velocity/stagnancy, which is one of the major triggers of DVT. RBCs may also enhance thrombin generation (reviewed in (Byrnes and Wolberg, 2017)). Polyhedrocytes, which are RBCs compressed within thrombus by surrounding contracting platelets adhered to fibrin fibrils, contribute to "sealing" the thrombus making it impermeable (Tutwiler et al., 2018). This makes hemostasis more efficient but can also limit the effect of thrombolysis thus supporting thrombosis (Byrnes and Wolberg, 2017).

Thus, RBCs, which have traditionally been considered "passive bystanders", play a substantial role in thrombosis.

7. Mast cells

In 1999, Bankl et al. demonstrated accumulation of mast cells in the adventitia of the thrombosed vein in DVT and suggested their profibrinolytic phenotype (Bankl et al., 1999). A study in patients with urticaria pigmentosa (chronic accumulation of mast cells in the skin) showed prolonged bleeding time in wounds made on skin lesions, which also implies that mast cell might be anti-thrombotic (Kauhanen et al., 1998). Later works, however, demonstrated possible opposite role of mast cells. Stabilization of mast cell membrane prevented experimental thrombosis induced by diesel exhaust particles (Nemmar et al., 2004). In experimental DVT induced by IVC stenosis, two strains of mast cell-deficient mice were completely protected against DVT (Ponomaryov et al., 2017). Pharmacological stabilization of mast cells also reduced the prevalence of thrombosis. Surprisingly, histamine inhibitors

did not protect mice from DVT.

Mechanisms of mast cell-mediated thrombosis are still to be determined. It is possible that histamine released from mast cells, contacts abluminal side of the endothelial cells, and that is why systemically administered histamine inhibitors (affecting their luminal side) were ineffective. The level of endothelial activation sufficient to initiate thrombosis may require more than one hit (e.g., histamine and tumor necrosis factor-α combined). Mast cells also contain polyphosphate, a substance that was recently demonstrated to have potent procoagulant and proinflammatory activity (Mailer et al., 2019; Morrissey and Smith, 2015). Mast cells can also release extracellular traps and the presence of mast cell-derived traps was reported in coronary thrombi (Pertiwi et al., 2019; von Kockritz-Blickwede et al., 2008). Similar to NETs, mast cell-derived extracellular traps might stimulate thrombosis.

A substantial number of immune factors implicated in DVT have been described (Budnik and Brill, 2018). We hypothesize that mast cells could be the primary censor of flow stagnation and thus orchestrate further events and mediators involved in thrombosis initiation.

8. Conclusion

It is increasingly clear that bone marrow-derived cells are involved in different stages of DVT from thrombosis initiation to resolution. The role of these cells may differ depending on the experimental model in question, and therefore, caution should be exercised in the extrapolation of results obtained in the lab to clinical practice. However, bone marrow-derived cells and processes related to them can represent promising targets for DVT prevention. Identification of precise mechanisms of stasis/hypoxia-induced mast cell activation as well as mast cell-derived mediators that trigger thrombosis could pave the way to development of a brand new approach to antithrombotic therapy. Targeting NETs and, potentially, extracellular traps from other cells, by either inhibiting their formation or inducing destruction, may open new opportunities to DVT prophylaxis. Finally, further studies of the role of platelets in DVT, including specifically targeting procoagulant platelets, will allow for more efficient targeting of these cells in venous thrombosis.

Declaration of Competing Interest

The authors report no declarations of interest.

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