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Plasma DNA and Neutrophil Extracellular Traps (NET): A Novel Biomarker in Lower Limb Venous Thrombosis

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Abstract

The traditional teaching of the pathophysiology of thrombosis centers around platelets and the coagulation cascade. The role of neutrophils in the thrombotic process is an area of recent interest. This role of neutrophils in this novel process of cell death and formation of neutrophil extracellular traps (NETs) has been linked to multiple disease processes like vasculitis, sepsis, cancer and haematological disorders in which a diagnostic and therapeutic role are being rapidly elucidated. Deep vein thrombosis and pulmonary embolism is a major worldwide health concern with significant mortality and morbidity. The role of NETs in lower limb thrombosis is under study. In this paper, we review the need for a good biomarker in DVT, the role of NETs in thrombosis focussing on lower limb venous thrombosis.

Keywords: Plasma DNA; Neutrophils; NET; Biomarker; Venous thrombosis; Limb; DVT

Introduction

Deep vein thrombosis (DVT) is a major health care problem all over the world [1,2]. The population incidence can be as high as 1 in 100 individuals [3]. Lower limb DVT is associated with disease specific acute (pulmonary embolism) and chronic sequelae (post thrombotic syndrome) which cause significant morbidity. DVT is also associated with high all-cause mortality [4,5]. This has led to the recognition of the need for clinical and basic research in this field.

Clinical problems in diagnosis and treatment

Current practice uses probability scores and objective imaging techniques such as compression ultrasonography, Computed tomography (CT) venography or MR venography to confirm diagnosis [5-7]. Even though widely available, all have potential limitations. Pitfalls in venous duplex imaging include operator dependence, misidentification of veins, duplicate vein systems, systemic illness or hypovolemia resulting in decreased venous distention and areas not amenable to compression such as iliac veins, the femoral vein at the adductor canal, and the subclavian veins in 1% to 6% of patients [5,7-9]. Patients in ICU's with a diagnostic dilemma about DVT/PE may be unstable-precluding the possibility of confirmatory imaging. Suboptimal imaging is a common problem in obese or edematous patients, patients with pelvic fractures, large external fixators or multiple limb fractures also pose diagnostic challenges. Venography is expensive and though the traditional gold standard is of limited clinical utility and other imaging modalities have an appreciable false negative rate [5]. Clinical risk scoring models like the Wells, Geneva and Caprini scores were developed to overcome these limitations [5]. These were used in combination with D dimer; however this biomarker had a low specificity in the presence of other medical conditions [5].

Though there have been significant advances in the overall understanding of DVT, much remains unknown about the etiopathogenesis and outcomes in specific scenarios like trauma, DVT in children, systemic thrombosis, vasculitis associated thrombosis and cancer associated DVT.

Inflammation and thrombosis is linked

Starting around the late 90's; newer insights into the role of systemic inflammation and infection in venous thromboembolism (VTE) were identified [10-17]. This was accompanied by significant advancements in the understanding of the molecular mechanisms and the realisation that DVT too was a systemic process and not as distinct from arterial thrombosis as previously assumed [18-25]. Several studies in human and animal models identified a wide range of proteins, receptors and other substances distinct from the traditional coagulation cascade that were involved in the process of thrombosis [25-28]. In parallel to attempts at risk prediction in sepsis, a wide variety of biomarkers like CRP, P-selectin, microparticles, E-selectin, thrombin, interleukins and fibrin monomers were studied for clinical risk prediction and diagnosis. However, these were reported to have varying sensitivity (54-71%) and specificity (62-81%) with an overall accuracy of 60-76% [29]. Though these did not find widespread clinical applicability; they helped in furthering the basic science behind the process of thrombosis.

The traditional understanding of the process of thrombosis centred around the production and release of tissue factor by platelets, conversion of pro-thrombin to thrombin, fibrinogen to fibrin and formation of platelet thrombi. The understanding that platelets and neutrophils function in concert in the thrombotic process has been known since the 70s [30]. Clinical correlation between inflammation and thrombosis has also been described in many clinical scenarios like endotoxemia, vasculitis and inflammatory bowel disease and other conditions known to be risk factors for DVT [31-33].

Platelets and neutrophils link inflammation, immunity and thrombosis

From this time, there has been a lot of basic research on the relationship between inflammation and venous thrombosis that has led to a better understanding of the multiple role of platelets linking thrombosis, inflammation and immunity [31-34]. Platelets facilitate

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neutrophils adhesion, cytokines release to activate leukocytes, induce endothelial expression of adhesion molecules and release of soluble mediators, to induce direct killing of infected target cells [35]. They thus and modulate the endothelium, neutrophils, and lymphocytes initiating inflammatory and immune responses. Platelets also encase pathogens within platelet aggregates, and enable their direct internalization. One of the most significant advances in the understanding of DVT has thus been the elucidation of the hitherto unknown role of neutrophils in the process described as the formation of neutrophil extracellular traps and NETosis [39]. The role of NETs in thrombosis continues to be defined. In the following sections we will review the current advances in the basic science and clinical understanding of this process specific to lower limb thrombosis.

Neutrophil Extracellular Traps (NET) history

The role of neutrophils in the thrombotic process was first described in 1971 but received better attention around the early 2000's when it the role of neutrophils was reported in experimental dysentery, spontaneous human appendicitis, and animal models of lung injury. NET release was first described by Brinkmann et al.

NET structure

Neutrophils stimulated with microbes or pro-inflammatory agents, reactive oxygen species (ROS) or activated platelets, release their nuclear material, forming a web-like extracellular network. These webs, formed by DNA, histones and neutrophil granule constituents are designated as neutrophil extracellular traps (NETs) Formation of NETs is a step-wise process characterized by nuclear membrane dissolution, chromatin de-condensation and cytolysis driven by the activation of the enzyme peptidylarginine deiminase (PAD4) [40]. NET formation is thus associated with a cell death process distinct from apoptosis or necrosis. This has been termed NETosis [40]. This functions to kill a range of pathogenic bacteria, fungi and viruses. NETs can form both in tissues and in blood vessels, thus plasma DNA level increases in this condition [41]. NETs can also trap thrombi and the complete range of their functions is not understood yet.

NET clinical studies in systemic diseases related to thrombosis

Plasma DNA has been found to a biomarkers for sepsis [40-41]. Plasmas DNA is elevated during active vasculitis (SLE, RA, ANCA, APLA) which correlates to high thrombotic risk. High levels of NET and plasma DNA has been shown to predict adverse events in patients with coronary artery disease, transfusion related injury, thrombotic microangiopathies (TMA), thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, and malignant tumor induced TMA. NETs form in infection, endotoxemia, vasculitis, cancer and inflammatory bowel disease which are known to predispose to thrombosis [41-48]. Other conditions predisposing to DVT (trauma, surgery, immobilization, hypoxia) are also associated with NET formation [49]. NET formation and NETosis is the predominant role of neutrophils in venous thrombosis.

The formation of NETs causes release of cell free DNA into blood and tissue; thus NETs are detected by DNA measurements from serum or demonstration of extracellular DNA by immunocytochemistry. (50—61) The measurement of DNA in blood (plasma DNA) is the more clinically useful method.(61) The latter is difficult in DVT due to the clinical problems in securing tissue samples. Other different approaches to identifying NETs in blood and tissue are under study. Plasma DNA may be elevated in other conditions like sepsis, inflammation or tumors; but it has been reported to have a high specificity in clinical studies as discussed in a later section. (61) Whether this find wider applicability remains to be studied.

Net Role in Thrombosis

Animal models

Production of NET's has been studied in the inferior vena cava thrombosis model in PAD4-knockout mice and iliac vein of baboons. Studies reveal higher plasma DNA and NET levels in venous thrombi; thrombus size/numbers are larger with higher VWF, platelet activation and recruitment. Cleavage of NETs by DNase1or heparin aborts thrombosis and histone infusion can accelerate the process [49-51]. The role of NETs in relation to shear stress, vessel size, platelet plug and type of vasculature is yet to be investigated [51].

Human studies

The exact role of NETs in DVT pathophysiology is not completely elucidated. The simplistic concept of stasis, vessel wall damage and hypercoagulability has undergone much refinement with a better understanding of thrombus formation, platelet aggregation and clotting factors and formation [51-52]. NETs are involved in almost all steps of the thrombotic process.

Endothelial activation by hypoxia or other triggers and the release of VWF stimulate NETosis. Other processes like calcium flux or proteolytic receptor activation may further this cascade; causing more NET release and worsening endothelial damage [53,54]. It also promotes further platelet entrapment aggregation and activation via specific receptors [toll-like receptors, vWF, fibronectin and fibrinogen] or electrostatic coupling [55]. The release of tissue factor (TF) from NETs, microparticles and other chemokines is followed by the formation of the factor TF-FVIIa complex [56-57].

This process of TF release from NETs has been shown to have important clinical correlations in sepsis and some authors suggest that this is an overshoot of a normal homeostatic process [57-58]. NETs are also involved in proteolytic cleavage of TF inhibitors and increase of Factor Xa activity [58,59]. They promote fibrin formation by stimulating factor XII [60].

Human clinical studies in venous thrombosis

Though significant, data on lower limb DVT is not extensive. One group has reported on the use of Plasma DNA as a marker for DVT with a sensitivity and specificity of 81% [61]. This is better than most current non-invasive tests; and needs larger trials to establish its clinical utility.

These findings were corroborated by another study where elevated levels of plasma DNA were present in patients with confirmed DVT in comparison to those with clinical suspicion but no DVT on confirmatory testing. This study also used plasma DNA as a marker of risk prediction in DVT [62]. Plasma DNA has to be shown to occur during the process of DVT including various stages of thrombus organization, not be present before the onset of thrombosis and therefore demonstrating a role in thrombus maturation [63,64]. The role of NETs in relation to thrombolysis and other treatment modalities are under study.

Conclusion

The role of neutrophils in the thrombotic process is a recent advance in the understanding of venous thrombosis. Neutrophils have an essential and unique; the formation of neutrophil extracellular traps (NETs) has been shown to have an etiological role in many diseases

associated with systemic inflammation. It is a potential biomarker in deep vein thrombosis and the post-thrombotic syndrome with a multiple therapeutic implications. It is a good clinical target for further research.

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