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Review article

Phosphatidylserine is an overlooked mediator of COVID-19 thromboinflammation

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ABSTRACT

A ubiquitous component of cell membrane, phosphatidylserine (PS), is likely to play a major, but as yet unrecognized, role in the thromboinflammation of COVID-19 and other critical illnesses. PS is present in all plasma membranes but is "hidden" on the inner surface by the action of an ATP-requiring enzyme. Failure of PS to be sequestered on the inner surface of cell membranes, release of PS-containing microparticles from cells, or shedding of enveloped viruses allows it to interact with extracellular proteins, including those of the coagulation and complement systems. Detection and quantification of circulating PS is not standardized, and current methodologies have either focused on circulating cellular elements or subcellular plasma components, but not both. PS may also promote thromboinflammation without circulating if expressed on the surface of endothelial cells, a condition that might only be documented if novel imaging techniques are developed. Research into the role of PS in inflammation and coagulation, called here a "procoagulant phospholipidopathy" may provide novel insights and therapeutic approaches for patients with a variety of illnesses.

1. Introduction

From the beginning of the COVID-19 pandemic, observers have noted a high incidence of thrombosis involving both the arterial and venous systems and a syndrome of COVID-19 associated coagulopathy, sometimes denoted as "thromboinflammation" has become recognized [1, 2]. Left unanswered is the question "Is COVID thromboinflammation a unique entity, or simply a more highly reported upon accompaniment of other critical illnesses"? Certainly other severe viral infections, including Ebola [3], hantavirus [4], Crimean Congo hemorrhagic fever [5], Marburg virus [6] and Dengue hemorrhagic fever [7] have been associated with coagulopathy. Perhaps the wide-spread recognition of COVID-19 coagulopathy is simply due to the fact that it has occurred in large numbers of people living in higher-income nations in an era where rapid dissemination of information is possible because of the internet, rather than a unique pathophysiology.

Observations of thrombosis detected both macroscopically (clinically) and microscopically have alerted many to the occurrence of endothelial infection and inflammation along with activation of the complement system [8, 9, 10]. Experts have quickly assembled lists of potential points of intervention and drugs that might target both the coagulation and complement systems, interrelated as they are in many types of inflammation [11, 12].

Absent from discussions of COVID-19 pathophysiology (or potential treatment) is a molecule long recognized to play a role in normal human physiology but under-recognized as a significant actor in pathophysiology, the phospholipid phosphatidylserine (PS). A ubiquitous component of the inner surfaces of plasma membranes, PS is always present at sites of inflammation and cell death, but is never considered to be an active participant in causing tissue injury.

2. Phosphatidylserine, coagulation and thrombin generation

PS is one of the more abundant phospholipids of plasma membranes but is only found on the inner surface of plasma membranes under normal conditions. This is due to the action of an ATP-dependent "flippase", an enzyme that quickly moves any PS that diffuses to the external plasma membrane, back to the cell's interior [13, 14]. Upon activation or injury, cells may "expose" PS on their external surfaces, which allows coagulation proteins to gather onto a previously "non-thrombogenic" membrane surface, interacting in the coagulation cascade to generate the enzyme thrombin. Thrombin is a key enzyme in the thromboinflammatory process, not only because it generates fibrin clots but because a number of cells known to be involved in inflammation express thrombin receptors, including platelets, neutrophils, endothelial cells,

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macrophages, monocytes, smooth muscle cells and fibroblasts (reviewed in [15]). Thrombin generation may play an important role in both acute COVID-19 infection as well as the chronic problems affecting a subset of infected individuals.

Although the exposure of PS on intact platelet surfaces has been most intensively studied, PS exposure also occurs on platelet fragments ("microparticles") and on the surface of a variety of other cells and cell-derived microparticles, including erythrocytes [16, 17], tumor cells [18, 19], neutrophils [20], monocytes [21], and endothelial cells [22, 23]. PS may also be exposed on the surface of cells undergoing "programmed cell death" (apoptosis) as well as other types of cell death [24, 25] (Figure 1). Students of blood coagulation have concluded that exposure of PS is a major regulator of the blood coagulation system [26]. Despite a vast literature on "hypercoaguable states", both congenital and acquired, PS remains unrecognized by those who itemize the list of potential participants in thromboinflammation. In some clinical settings procoagulant phospholipids may play an active role in promoting tissue injury, inflammation and thrombosis, a condition that might appropriately be called a "procoagulant phospholipidopathy". As with other intracellular molecules such as nucleotides [27], DNA [28], histones [29], polyphosphates [30], RNA [31], and actin [32] that play a role in pathophysiologic states, procoagulant phospholipids may not be easily quantified in circulating blood from healthy subjects.

3. PS exposure in the blood due to endothelial infection by enveloped viruses

Enveloped viruses are so named because they exit from host cells by the process known as "budding", wherein the viral genetic material leaves the host cell with a coating ("envelope") of plasma membrane. In many severe viral infections involving enveloped viruses, the infectious particles themselves likely mediate activation of the coagulation system directly (rather than simply stimulating a host inflammatory response) because the PS in those envelopes is not maintained on the interior of the viral particle [33]. The "exposed" PS thereby provides a surface for assembly of plasma coagulation proteins, which generate thrombin, as first shown for cytomegalovirus [34] and subsequently HIV-1 [35]. It is likely that this occurs with other severe viral infections accompanied by coagulopathy and hemorrhage. Further, "exposed" PS facilitates viral infection of some cells [36]. Targeting of PS in such infections has been proposed as a therapeutic goal [37].

The virus responsible for the COVID-19 pandemic is an enveloped virus whose main portal of entry into a variety of cells is the protein called angiotensin converting enzyme 2 receptor (ACE2R) (reviewed in [38]). This protein is found on many cell types, including epithelial cells, endothelial cells and leukocytes. Its expression on the surface of the endothelial cells means that the release of viral particles from the infected cells introduces PS-expressing particles directly into the blood stream rather than the extravascular space. It is currently unknown what proportion of PS released from other types of cells might also find its way into the circulation from the interstitial space. Given the wide-spread organ dysfunction that is seen in some patients with COVID-19 infection, release of PS from non-endothelial cells is likely to be clinically significant in some patients.

4. Measuring circulation phosphatidylserine (PS)

Two methods have been used to detect circulating PS in blood, but neither has been perfected and there is no agreed upon "gold standard" for either approach. Most of the published literature has used flow cytometry to detect microparticles (also called microvesicles), which can be found in the circulation of patients with a variety of diseases including cancer [39], as well as kidney [40], lung [41] and cardiovascular diseases [42, 43]. Importantly, detailed analysis of platelet-derived microparticles has shown that all microparticles are not equally thrombogenic [44]. Standardization of detection and reporting of microparticles (only some of which are pro-thrombotic) is an important area of investigation [45].

Blood coagulation tests have been adapted to detect circulating PS [46, 47, 48]. Such tests have not been widely adopted, in large part because of a lack of appreciation of the importance of PS in human disease but also because they are technically demanding and require almost immediate testing of collected specimens to avoid artifactual "exposure" of PS on blood cell or fragments after venipuncture. Such tests are typically performed on plasma, which can be prepared by any of a number of centrifugation maneuvers, but the details of centrifugation (often taken for granted in clinical studies) may itself influence the apparent amount of circulating PS [46, 49]. Importantly, methods of measuring circulating PS in whole blood have not been developed, and all published studies to date have excluded circulating blood elements.

5. How could recognition of PS's role in pathophysiology improve health care?

Treatments to ameliorate PS exposure would be a clear avenue to explore. Since PS is known to be "neutralized" (from a coagulation standpoint) by proteins such as annexin V [50] and lactadherin [51] it

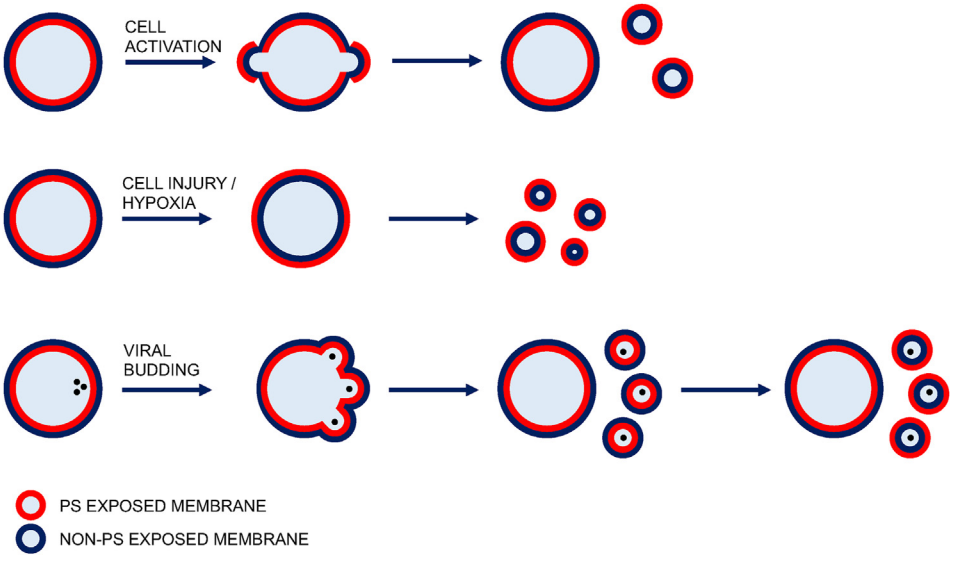

Figure 1. Phosphatidylserine exposure on the outside of cells and microparticles – Phosphatidylserine (PS) is maintained on the inner surface of the plasma membrane of all cells. Several mechanisms can lead to the "exposure" of PS on the external surface of cells. Physiologic or pathologic "activation" of cells can lead to exposure of phosphatidylserine (PS) on the external surface of cells, or microparticles released by cells. Enveloped viruses bud from host cells, resulting in PS-bearing virions released into the intra- and extra-vascular spaces, depending upon the location of the infected cell.

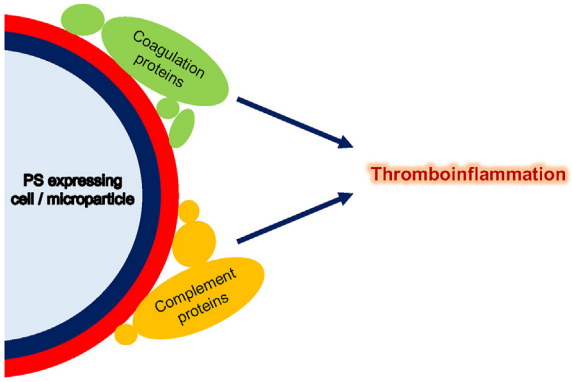

Figure 2. Phosphatidylserine exposure on the surface of cells exposed to plasma results in thrombin generation and complement system activation - Blood coagulation proteins have phosphatidylserine-binding domains, which allows them to cluster together on exposed PS, accelerating their interaction and the subsequent generation of thrombin. PS can lead to activation of the complement system. Together, the coagulation and complement systems contribute to the complex clinical phenotype of thromboinflammation.

would be logical to explore their use as therapeutics. Unlike lactadherin which is glycosylated, annexin V (a circulating human plasma protein [52]) is not, and can be produced by bacteria as a monomer, dimer [53], or chimeric fusion protein [54, 55]. Annexin V has been utilized for imaging and drug delivery [56] and is an attractive [57, 58] (though perhaps hard-to-patent) candidate for developmental therapeutics. Alternatively, monoclonal antibodies to phosphatidylserine have been produced [59] and proposed as potential anti-viral agents [37]. Since many proteins, notably including those involved in blood coagulation, have PS-binding domains, protein engineering of high-affinity, non-immunogenic proteins would be another avenue to pursue.

Novel treatments that are only based upon tests of circulating procoagulant PS run the risk of overlooking possible benefits of endothelial-directed PS-neutralizing therapies. Endothelial cells express receptors for SARS-CoV-2, and endothelial cell budding of the virus may result in endothelial surface expression of PS. Some therapies might prove to be useful by targeting PS on endothelial surfaces, even in the absence of circulating PS. Imaging of vascular beds may be useful in documenting PS-exposing endothelium, since the surface area of the vascular system is quite large [60]. Development of such imaging modalities could be used not only to explore the contribution of the endothelium to thrombosis in severe infections, but other conditions where endothelial dysfunction may contribute to pathophysiology, such as acute kidney injury or systemic hypotension. Studies with an engineered annexin V dimer, for example, suggest its potential for improving health, even in the absence of proof of high levels of circulating PS in targeted disease states [61, 62, 63].

There are a number of additional reasons for the research community to turn its attention to the importance of "exposed" (i.e. procoagulant) PS: 1) there is a documented interaction between PS and the complement system, shown to be activated in COVID-19 and likely other infectious states [64]. Complement-mediated injury of cells leads to exposure of procoagulant PS on the cell surface, thereby promoting thrombosis [22, 65] (Figure 2). Further, exposure of PS leads to activation of the complement system, as shown with apoptotic cells [66]; 2) Immune suppression is recognized to accompany exposure of apopotic (i.e. PS-bearing cells) to macrophages [67], and may play a role in the development of secondary infections in patients with COVID-19 and/or other critical illnesses [68]; 3) several plasma proteins bind to PS, and some may play a protective role in neutralizing the effect of PS upon coagulation and inflammatory pathways. Provision of these proteins by plasma infusions may be useful for future pandemics before specific antibodies are available for treatment or may explain beneficial effects of plasma infusions that do not contain appreciable amounts of antibody; 4) exaggerated release of PS-containing microparticles from platelets, neutrophils and monocytes of diabetic patients [69] may explain, in part, the increased susceptibility diabetic patients to COVID-19; and 5) PS may be an unrecognized but active contaminant of many biological preparations [70].

6. Summary

PS may be an overlooked actor in many inflammatory states and should become the subject of more robust and widely available testing. Inflammation always involves multiple mediators and regulatory pathways, and it is unlikely that a single molecular entity (or novel blood test) will fully explain the wide spectrum of host responses in complex disease states. In fact, therapeutic interventions may ultimately be required to define which of many possible mediators are active in a given sub-group of patients. Increasingly, molecules that are familiar to us because of their defined roles in intracellular biochemistry (such as ADP, DNA, histones, polyphosphate, and actin) are being found to play important extracellular roles in a variety of pathophysiologic states. Phosphatidylserine (PS) should be added to this list so that it too is considered when multidisciplinary approaches to critical illness are considered. A "procoagulant phospholipidopathy" may emerge as a heretofore unrecognized mediator of morbidity and mortality in several disease states.

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