The central role of neutrophil extracellular traps in SARS-CoV-2-induced thrombogenesis and vasculitis

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Abstract

Objective: Severe acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) seen in SARs-CoV-2 infection has been attributed to the disruption of the immune response in COVID-19 patients. Neutrophilia and marked lymphocyte reductions are associated with disease severity and seem predictive of disease outcome in moderate and severe COVID-19 patients. Herein, we aim to decipher possible mechanisms involved in extensive tissue injury observed in COVID-19 patients, accompanied by vasculopathy, coagulopathy, and a high incidence of thrombotic complications in severe patients.

Methods: We searched PubMED for keywords including COVID-19 pathogenesis, thrombosis, and vasculities.

Results: Neutrophils can undergo a specialized form of apoptosis to yield thread-like extracellular structures termed neutrophil extracellular traps (NETs), termed NETosis, which form web-like scaffolds of DNA, histones, and toxic protein granules and enzymes, whose primary function is to trap and eliminate microbes. However, uncontrolled NET production can lead to ALI and ARDS, coagulopathy, multiple organ failure, and autoimmune disease. Dysregulation of NETs promotes production of anti-neutrophil cytoplasmic antibodies (ANCA) which affects small vessels through ANCA-associated vasculitis (AAV). Furthermore, NETs can also induce thrombosis via formation of scaffolds that trap platelets, RBCs, fibronectin, and other proteins, which can also induce coagulation.

Conclusion: We suggest that NET production is central during SARS-CoV-2 infection and COVID-19 pathogenesis, associated with alveolar damage accumulation of edema, endothelial injury and coagulopathy, elevated platelet activation and thrombogenesis forming a NET production feed-forward loop, causing diffuse small vessel vasculitis in the lungs and other organs.

Key words: COVID19; Coronavirus; Neutrophils; Neutrophil extracellular traps (NETs); SARS; Thrombosis; Vasculitis

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Introduction

Severe acute lung injury (ALI) seen in SARs-CoV-2 infection seems attributable to the disruption of the inflammatory and immune response to viral infection.[1] The acute respiratory distress syndrome (ARDS) observed in COVID-19 patients presents a distinct phenotype, whereby patients develop profound hypoxemia early in the disease with minimal respiratory dysfunction.[2] Blood analyses also indicate neutrophilia, with a marked reduction of lymphocytes, particularly CD-8+ cells.3 Indeed, lower levels of CD-8+ cells are associated with disease severity, whereby ratios of CD-8+ cells/neutrophils and neutrophils/lymphocytes seem predictive of disease outcome.[3] High levels of proinflammatory cytokines including IL-6, IL-1 β, IL-10, and TNF-α have been noted in moderate and severe COVID-19 patients.[4,5] Collectively these findings suggest that neutrophilia with an associated moderate rise in cytokine levels is core to disease pathogenesis. However, this does not explain the extensive tissue injury seen in these patients.

Several studies suggest the occurrence of vasculopathy and coagulopathy accompanying the cytokine level elevation as contributing factors in COVID-19 pathogenesis, while a high incidence of thrombotic complications in critically ill patients with this disease have also been increasingly noted.[6,7,8] Indeed, >40% of hospitalized COVID-19 patients showed thrombotic complications.6 Segmental hyperperfusion with vasodilation and endothelial dysfunction in the pulmonary vasculature of COVID-19 patients has been observed, while further reports indicate increases in pulmonary dead-space attributable to pulmonary micro-thrombosis and embolism.[9]

Neutrophil Extracellular Traps (NETs) are web-like structures consisting of DNA, histones, and enzymes such as Myeloperoxidase (MPO), that released from neutrophils as they undergo a specialized type of cell death called "NETosis", and are increasingly being associated with key roles in COVID-19 infection and disease pathogene-

sis.10 Although the primary function of NETs is to trap microbes and associated debris, an uncontrolled proliferation of NETs from neutrophils culminates in alveolar damage, endothelial injury and coagulopathy.[10] Indeed, MPO-DNA and citrullinated histones, NET-specific markers, are significantly elevated in COVID-19 patient sera.[11] Additionally, cell-free DNA is strongly correlated with levels of D-dimer, lactate dehydrogenase and acute reactant proteins like C-reactive proteins in such patients.[11] Interestingly, sera from COVID-19 patients triggered NET production in healthy control-derived neutrophils.[11] It is thus possible that ALI, vasculopathy, and coagulopathy seen in COVID-19 patients could be due to excessive release of NETs from neutrophils.

Specialized pro-resolving mediators (SPMs) derived from omega-3 polyunsaturated fatty

acids are key in controlling pro-inflammatory collateral tissue damage and vasculopathy caused by excessive cytokine and NET levels.[12,13] To this degree, SPMs such as resolvins could mitigate uncontrolled inflammatory responses observed in COVID-19 patients.[12,13] Herein, we hypothesize that: ALI, vasculopathy and coagulopathy in severe COVID-19 patients is due to an uncontrolled inflammatory response of neutrophils, resulting in moderately high levels of cytokines and excessive release of NETs. We propose that this could be effectively controlled by exogenous administration of SPMs such as resolvins.

Materials and Methods

We reviewed the scientific literature through PubMed by looking for terms including COVID-19, NETosis, NETs, Coagulopathy, Vasculitis and resolvins. We also evaluated literature to understand the cellular processes involved in NETosis which can lead to coagulopathy and vasculitis and how resolvins can regulate uncontrolled NETosis.

Results

Neutrophils are the most abundant cell type in circulation, and are crucial players in the innate immune response against pathogens, alongside other various functions in both infectious and non-infectious disease pathogenesis.[14] Recently, the expulsion of chromatin by neutrophils to form specialized structures known as neutrophil extracellular traps (NETs) has gained attention in describing the function of neutrophils in health and disease.[15] Brinkmann et al first described NETs as web-like scaffolds of DNA, histones, and toxic protein granules, which primarily functioned to trap and eliminate microbes.[16] Although NET release during infections is physiologically beneficial, excessive NET production could prove harmful, resulting in tissue injury and thrombosis.[17,18]

NETosis is a well-orchestrated process, whereby neutrophils undergo morphological alterations in response to various stimuli including infections, platelets, and inflammatory mediators, resulting in the loss of neutrophil nuclear lobulation in the with subsequent swelling and nuclear membrane rupture.[19,20] This results in mixing of nuclear components (DNA and histones) with cytoplasmic granular content including myeloperoxidase (MPO) and neutrophil elastase (NE).[19,21] An established mechanism of NETosis is citrullination of histones by enzyme peptidylarginine deaminase-4 (PAD-4), which citrullinates histones H3 and H4, resulting in chromatin decondensation and nuclear swelling.[18] In mice, absence of PAD-4 prevented NET formation, while calcium ionophores could activate PAD-4 resulting in NETosis.[22,23] Furthermore, NADPH oxidase complexes are also able to stimulate NET production in PAD-4 independent manner.[19] The role of neutrophils in thrombosis is currently poorly understood. Neutrophils and platelets are the first cells recruited at inflammation or infection sites.[18] Neutrophils limit microbial spread by promoting coagulation via fibrin deposition.[24] In liver microvasculature, NET formation and fibrin deposition can prevent systemic spread of bacteria, while disruption of NETs promoted systemic dissemination.[24,25] Furthermore, an excessive activation or dysregulation of neutrophils in blood vessels can lead to coagulopathy, indicating a central role in the regulation of thrombosis through NETs.[15,26] NETs can potentially induce thrombosis via several mechanisms. NETs form scaffolds which traps platelets, RBCs, and platelet adhesion molecules such as fibrinogen, von Willebrand factor (vWF) and fibronectin.[27] Such scaffolds not only provide the structural basis for thrombosis, but can also induce coagulation through its various constituents by activation of platelets.[28] Strikingly, individual components of NETs are more potent inducers of thrombosis than a complete NET complex.[29] Histones H3/H4 cause platelet activation directly via Toll like receptor (TLR) 2 and 4, and by interacting with fibrinogen, and cause platelet aggregation, proving toxic to endothelial and epithelial cells. Histones bind thrombomodulin, preventing activation of activated protein (APC), further increasing thrombogenesis.[30,31] Furthermore, serine proteases in NETs can activate both intrinsic and extrinsic coagulation pathways.[24] Interestingly, activated platelets can stimulate neutrophils to produce NETs, thus establishing a feed-forward loop, potentially exacerbating disease conditions.[32,33] Indeed, NETs form an integral part of thrombi in histological examination of tissues derived from patients with myocardial infarctions and strokes.[34-36]

Dysregulation and excessive production of NETs can promote the production of anti-neutrophil cytoplasmic antibodies (ANCA), which are associated with a type ANCA-associated vasculitis (AAV) which affects small vessels and it is accompanied by elevated ANCA levels in patient serum.[37,38] Excessive NET production or a reduction in NET degradation can lead to formation of MPO-ANCA

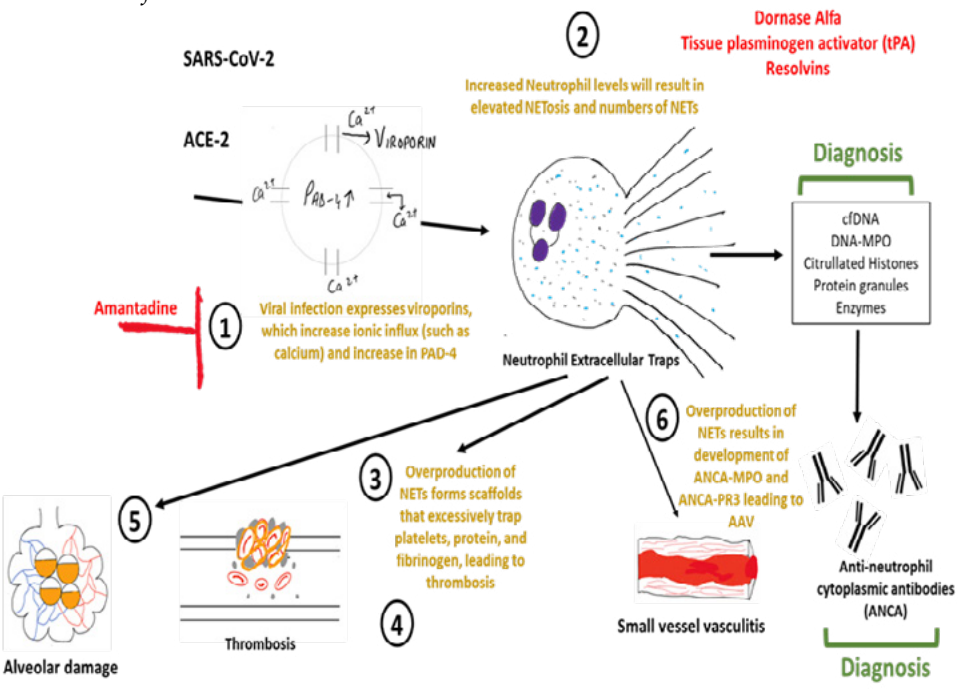
and PR3-ANCA mediated via DNA deposits in NETs.[39,40] The pathogenesis of AAV is sequential, involving 1) release of pro-inflammatory cytokines IL-1 β and TNF-α by macrophages leading to neutrophil activation, 2) expression of neutrophilic antigens MPO and PR3 on neutrophil surfaces, 3) ANCA-antigen binding resulting in neutrophil hyperactivation, and 4) excessive cytokine, reactive oxygen species (ROS), and NET release by neutrophils leading to endothelial cell injury.[37]

Classically, inflammation can be divided into two distinct phases. The first is initiation, involving a swarming of neutrophils to the inflamed area due to pro-inflammatory cytokines. The second phase is resolution, involving a decrease in levels of pro-inflammatory mediators.[41] However, resolution is no longer thought to involve a passive decrease of inflammatory mediators, but rather is now considered an active process, requiring lipoxins released from arachidonic acid stop signals for the infiltration of neutrophils.[42] Three classes of chemical mediators termed specialized pro-resolving mediators (SPM) derived from lipoxins have been classified, which are involved at inflammation resolution; 1) resolvins (resolution phase interaction products) 2) protectins, and 3) maresins (macrophage mediators in resolution of inflammation).[13]

Discussion

SARS-CoV-2 infection leads to elevated cytokine levels such as IL-6, IL-1 β, and TNF-α. However, this seems to be only a moderate rise and do not correspond with the pathogenesis of ARDS observed in COVID-19 patients.[6] Thus, perhaps the initial rise in cytokine levels results in swarming of neutrophils to the inflamed lung. Subsequently, SARS-CoV-2 infect neutrophils via ACE-2 receptors and induce calcium influx through expression of viroporins on the cell surface. High intracellular calcium leads to activation of PAD-4, which translocate into the nucleus, resulting in chromatin decondensation, nuclear membrane rupture, and release of NETs. We hypothesize that excessive NET release from neutrophils is central SARS-CoV-2 infection and COVID-19 pathogenesis. Such excessive NET release has been associated with alveolar damage and accumulation of edema, endothelial injury and coagulopathy, elevated platelet activation and thrombogenesis forming a NET production feed-forward loop, and finally development of AAV due to production of NET constituent associated ANCAs, causing diffuse small vessel vasculitis in the lungs and other organs (Figure 1).[10, 11, 18, 37, 43-46]

Figure 1: *Schematic visualisation of the proposed mechanism underlying increased Neutrophil Extracellular Trap (NET) production in response to SARS-CoV-2 infection, leading to anti-neutrophil cytoplasmic antibodies-associated vasculitis (AAV) via NETs, as well as thrombosis, and subsequent alveolar damage (steps 1-6). Viral infection increases ionic influx through expression of viroporins, increasing PAD-4 levels. Elevated levels of NETs and their constituents will promote trapping of platelets, platelet activation and an activated platelet -NETs feed-forward loop. Elevated NETs also culminate in overproduction of ANCAs, leading to AAV. Concurrently, elevated NETs form large levels of scaffolds that excessively trap platelets, protein, and fibrinogen, leading to thrombosis. Potential candidates (red text) are displayed in appropriate areas where they can inhibit overproduction of NETs (red lines). Potential biomarkers are also indicated by green text and lines to indicate which candidates could be used as cell-free sera-based prognostic evaluators of COVID-19.*



Considering our hypothesized mechanism of pathogenesis of COVID-19 which remains as yet poorly understood, sera and bronchoalveolar lavage (BAL) of COVID-19 patients need to be evaluated for levels of NETs. Specific biomarkers for NETs in body fluids include cell free DNA, MPO-DNA, citrullinated histone H3 (Cit-H3) which can easily be detected by rapid (10-15 minutes) ELISA-based or strip assays.[11] In addition, ANCA-MPO and ANCA-PR3 should be assessed to rule out AAV caused by excessive NETs production.[37]

Conclusion

In light of the current evidence, therapeutic strategies should be developed to control the effects of uncontrolled NET release. One option could be use of recombinant DNase-1 (dornase alfa) which has been used previously along with tissue plasminogen activator (tPA) for resolution of thrombi caused by excessive NET production. Amantadine can block viroporins and calcium influx which is crucial for PAD-4 activation. Finally, excessive inflammatory tissue damage, coagulopathy and vasculitis caused by uncontrolled release of NETs can be controlled by exogenous administration of resolvins.

Recommendation

In future we intend to investigate the serum levels of NETs in the clinical samples and study their association with thrombosis seen in COVID-19 patients. This will help us to identify newer therapeutic targets to treat complications associated with SARS-CoV-2 infection.

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