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STATEMENT / OPINION

Neutrophil Extracellular Traps Capturing SARS-CoV-2 in the Lung Tissue (Alveoli and Parenchyma) Cause Microthrombi

— A Strategy to Eliminate SARS-CoV-2 From the Circulation as Degraded Fibrin Clots —

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Background: It has been thought that neutrophil extracellular traps (NETs) and thrombosis exacerbate COVID-19, but, on the other hand, NETs are an important player in innate immunity. The precise roles of NETs and thrombosis in the course of COVID-19 have not been fully elucidated.

Methods and Results: The roles were investigated in the literature and a new theory was formulated. When neutrophils encounter SARS-CoV-2 in the lung tissue, they undergo NETosis and capture the virus. This capture is triggered by electrostatic interaction between histones in NETs and SARS-CoV-2; histones are highly positively charged, and viruses, including SARS-CoV-2, have a net negative charge under physiological pH. NETs that capture SARS-CoV-2 fall into alveolar capillaries through the collapsed endothelium to spare the lung tissue from the toxicity of NETs. NETs in the microvessels cause microthrombosis; positively charged histones induce the aggregation of negatively charged platelets, which leads to microthrombi. Microthrombi engulfing SARS-CoV-2 are consolidated into fibrin clots, which are eventually degraded by increased fibrinolysis and eliminated from the circulation.

Conclusions: This novel theory suggests that NETosis and microthrombosis are phenomena inevitably elicited in COVID-19, and in combination they are a system newly termed "NETombosis". Undegraded fibrin clots remaining in the microcirculation may be the cause of the sequelae, because they cause long-lasting circulatory failure in various organs.

Key Words: Histones; Microthrombosis; Netosis; Platelets; SARS-CoV-2

It has been thought that neutrophil extracellular traps (NETs)[1–3] and thrombosis aggravate COVID-19,[4,5] but NETs are an important player in innate immunity; they catch and kill pathogens[6,7] and inhibit their dissemination. To resolve this discrepancy, I researched the precise roles of NETs and thrombosis in the course of COVID-19.

Induction of NETosis by SARS-CoV-2

In response to various stimuli, neutrophils can release nucleic acids decorated with histones and granular proteins, a phenomenon named "NETosis".[6,8] Locally high and lethal concentrations of myeloperoxidase (MPO) and defensin contained in NETs participate in the killing of pathogenic microbes.[9] NETosis is induced by activation of Toll-like receptors (TLRs). Neutrophils possess TLRs 1, 2, 4, 5, 6, 7 (low expression), 8, 9, and 10.[10] It is reported that viable SARS-CoV-2 can directly induce the release of NETs by healthy neutrophils.[1] Therefore, SARS-CoV-2 is a neutrophil stimulant.

Large Amounts of Microthrombi and NETs in the Lungs of Autopsied Patients

Pathological studies of the lungs of patients who succumbed to COVID-19 showed extensive diffuse alveolar damage and thrombi in the microvessels.[11–15] Carsana et al. found microthrombi (containing platelets and fibrin) in the pulmonary microarteries in 33 cases (87%).[12] NET formation, however, was focused on by very few investigators.[1,16–19] Veras et al. observed a characteristic NET-like structure with extracellular DNA that colocalized with MPO and citrullinated histone H3.[1] Middleton et al. showed a NET-like structure in which histone $H3^+$ neutro-

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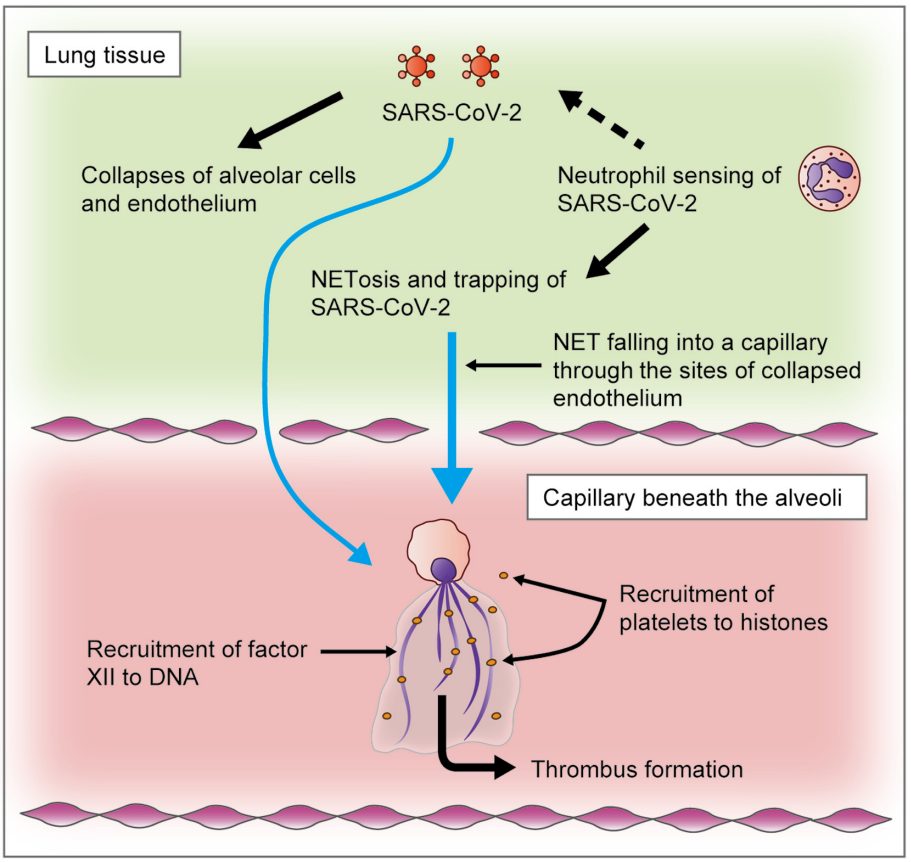

Figure. A process of microthrombus formation in COVID-19. SARS-CoV-2 invades the lung tissue, where infiltrating neutrophils sense it and cause NETosis. NETs (histones) trap SARS-CoV-2 via electrostatic interaction. These NETs fall into the alveolar capillaries through the collapsed endothelium to spare the lung tissue from the toxicity of NETs (positive charge of histones). A portion of SARS-CoV-2 evade NETs and directly fall into the capillaries. NETs in the microvessels generate microthrombosis, wherein the positive charge of histones triggers the aggregation of negatively charged platelets. DNA in NETs may partially participate in this microthrombosis; DNA recruits factor XII and initiates the intrinsic pathway. Microthrombi engulfing SARS-CoV-2 are consolidated into fibrin clots, which are eventually degraded by the elevated fibrinolysis and eliminated from the circulation. NETs, neutrophil extracellular traps.

phils colocalized with platelets in the lung; early-stage NET-forming neutrophils express citrullinated histone H3.[16] Radermecker et al. found NETs in a broadly scattered distribution in lung specimens.[19]

NETs Also Found in the Blood of Live Patients

The presence of NETs in the blood of live patients with COVID-19 has been confirmed by many investigators,[1,2,16] the levels of which were quantified by measuring the MPO–DNA complex or NETs showing both MPO and citrullinated histone H3. Overall, those studies revealed that patients with COVID-19 have significantly higher plasma NET levels than healthy individuals. Middleton et al. examined a marker for the severity of respiratory failure (PaO_2/FiO_2) and found that severely ill (intubated) patients had higher levels of plasma NETs, and nonintubated patients had lower levels of NETs.[16] This finding leads to 2 perspectives: (1) severely ill patients require higher levels of NETs to eradicate SARS-CoV-2, and (2) higher levels of NETs exacerbate the disease.

Positive Charge of Histones May Trigger SARS-CoV-2 Capture by NETs

NETs can effectively bind and capture a huge range of different microbes,[6] but the mechanism has not been sufficiently elucidated. I speculate that positively charged histones in NETs play a key role. Cationic antimicrobial peptides (AMPs),[20,21] such as defensins, cathelicidin (LL-37), and calprotectin, are present in NETs. These cationic AMPs are capable of bacterial, fungal, and viral killing.[22,23] Clogston and Patri reported that most cellular membranes are negatively charged.[24] Therefore, microbe killing by cationic AMPs may be triggered by electrostatic interactions. Two groups have reported that electrostatic forces between the cationic AMPs and the negatively charged bacterial surface are a critical determinant for their interactions.[21,25] Defensins can insert into and disrupt bacterial membranes, leading to bacterial lysis.[20] Similar to bacteria, viruses are negatively charged. Michen and Graule examined the isoelectric points of 44 species of viruses (including 132 strains).[26] The value ranged between 3.5 and 7.0, except for

3 strains, which indicates that almost all viruses have a net negative charge under physiological pH. Indeed, various viruses are targeted by cationic AMPs.[27] Concerning SARS-CoV-2, its zeta potential was found to be −25.675 mV,[28] which indicates that the viral surface is anionic. Clogston and Patri reported that zeta potentials > +30 mV or < −30 mV are considered strongly cationic and strongly anionic, respectively.[24] Accordingly, it is likely that SARS-CoV-2 interacts with cationic AMPs. Similar to cationic AMPs, histones have a high positive charge,[29] and thus presumably interact with SARS-CoV-2. Moreover, histones are a major NET constituent. Therefore, it is quite likely that histones in NETs play a critical role in SARS-CoV-2 capture by NETs. However, it has not been determined yet whether or not NETs can kill SARS-CoV-2.

NETs Cause Microthrombosis in the Microvessels of the Lungs

Silk et al. reported that histones seem to be released into the extracellular space in 3 forms: freely, as a DNA-bound nucleosome or as part of NETs, and all 3 forms can be detected in serum after significant cellular death.[30]

Extracellular histones induce microthrombosis. Fuchs et al. showed that purified histones, as well as NETs, cause platelet adhesion, activation, and aggregation when they are perfused with blood.[3] Accordingly, it is quite likely that NETs formed in the lung tissue cause microthrombosis when they fall into the capillaries beneath the alveoli. In more detail, neutrophils that have undergone NETosis by encountering SARS-CoV-2 in the lung tissue fall into the alveolar capillaries (**Figure**) through the endothelium collapsed by NETs or by viral cytopathic effects to spare the lung tissue from the toxicity of NETs (positive charge of histones); tissue toxicity of extracellular histones has been reported by many researchers. In the microvessels, positively charged histones in NETs bind to negatively charged platelets, which induces calcium influx into platelets and recruits plasma adhesion proteins such as fibrinogen, causing platelet aggregation by two mechanisms: αIIbβ3-dependent and -independent.[31] Neutrophil elastase and cathepsin G present in NETs potentiate platelet aggregation by proteolytically activating platelet receptors.[32,33] Such platelet aggregates develop into fibrin clots because coagulation factors may aggregate on the procoagulant surfaces of activated platelets, and consolidate them into fibrin clots.[34] DNA in NETs may also partially participate in the formation of microthrombi; DNA recruits factor XII and initiates the intrinsic pathway. Taken together, NETosis and the subsequent microthrombosis are a system to eliminate SARS-CoV-2, newly termed "NETombosis".

Elevated Fibrinolysis in COVID-19

In severe COVID-19, serum levels of D-dimers were mildly increased, while levels of FDP were extremely increased.[5,35] This indicates increased fibrinolytic disseminated intravascular coagulation in patients. Fibrinolysis is the proteolytic process of degrading fibrin clots, allowing their clearance from the circulation. Therefore, increased fibrinolysis, as observed in COVID-19 patients, indicates that fibrin clots containing SARS-CoV-2 are being removed significantly from the circulation. If such degradation becomes dysfunctional because of too many fibrin clots, circulatory failure will occur in the microvessels and cause functional disorders in various organs. This may be the cause of the sequelae of COVID-19.

Some NETs May Form in the Vasculature

A systematic literature review by Andersson et al. showed that SARS-CoV-2 RNA was detected in 0–76% of blood samples, with a pooled estimate of 10%.[36] Their study suggests that a portion of SARS-CoV-2 that invades the lung tissue evades NETs and fall directly into the alveolar capillaries. These SARS-CoV-2 probably cause NETosis in the vasculature, because it is known that NETs can be formed inside the vasculature in both infectious and noninfectious diseases.[37]

Differences Between NETombosis and Immunothrombosis

Both immunothrombosis[38] and NETombosis are systems to eliminate pathogens by enclosing them in microthrombi. However, the process is quite different between them. Immunothrombosis is evoked by tissue factor expression on monocytes, which is triggered by pathogens in the circulation, and initiates extrinsic coagulation pathway leading to microthrombosis. Because immunothrombosis is an event occurring in the vasculature, diseases that develop immunothrombosis are thought to be only bacteremia or viremia. In contrast, NETombosis mainly captures pathogens existing in extravascular tissues but also those in the vasculature.[37] Pathogens that evoke NETombosis are limited; they must cause NETosis. NETosis occurs when neutrophils encounter pathogens that they cannot kill by phagocytosis or by releasing toxic substances. At present, there are no diseases showing NETombosis other than COVID-19. However, I speculate that diseases in which NETosis develops may essentially cause NETombosis to remove tissue-toxic NETs. NETombosis comprises 2 processes. NETs released from neutrophils capture pathogens in extravascular tissues. Microthrombosis occurs after these NETs have fallen into microvessels. It should be noted that, in these processes, histones with an exceptionally high positive charge play a key role: the capture of negatively charged pathogens and aggregation of negatively charged platelets. Eventually, fibrin clots enclosing pathogens are formed and eliminated from the circulation by enhanced fibrinolysis.

Conclusions

NETombosis was created as a novel theory to explain the overall process of COVID-19. However, an important phenomenon, SARS-CoV-2 capture by NETs (histones), has not been experimentally confirmed yet. So, at present, NETombosis is a hypothesis. Nevertheless, it may give support to NETombosis theory that microthrombi have been found by histopathology in cutaneous eruptions of COVID-19 in 8 of 15 cases.[39] I hope this paper will help to extend our understanding of COVID-19.

Disclosures

The author declares no association with any individual, company, or organization in producing this manuscript.

Conflict of Interest

The author declares no conflict of interest.

IRB Information

Not applicable.

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