

Proteases, neutrophils, and periodontitis: the NET effect

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Neutrophils exert potent antimicrobial activities in their role as first-line cellular defenders against infection. The synergistic and collective actions of oxidants and granule proteins, including serine proteases, support the microbial killing in phagosomes, where most neutrophil-mediated antimicrobial action occurs. In addition to phagocytosis, specific stimuli prompt neutrophils to extrude a matrix of DNA, histones, and granule proteins to produce neutrophil extracellular traps (NETs), which can trap microbes. Mice lacking the serine proteases necessary for NET production are more susceptible to infection, an observation suggesting that functional NETs are required for host protection. In this issue of the *JCI*, Sørensen and colleagues characterize neutrophils from a patient with Papillon-Lefèvre syndrome. The patient has an inactivating mutation in the gene encoding dipeptidyl peptidase I, resulting in neutrophils lacking elastase, a serine protease required for NET production. Despite the inability to form NETs, neutrophils from this patient killed pathogens *in vitro*, and the patient did not exhibit evidence of an increased propensity toward bacterial infections. Together, these results suggest that proteases in human neutrophils are dispensable for protection against bacterial infection and that the ability to generate NETs *in vitro* does not compromise host defense.

He who studies medicine without books sails an uncharted sea, but he who studies medicine without patients does not go to sea at all.

—William Osler, MD

The desire to quell patients' suffering drives the quest to elucidate the mechanisms that underlie disease pathogenesis. Patients not only provide incentive for biomedical pursuits, but also redirect our notions about a particular disease when we drift off course. In this issue, Sørensen et al. describe the genetic abnormality in a young female with Papillon-Lefèvre syndrome (PLS) (1), a rare autosomal disorder also known as keratosis palmoplantaris with periodontopathia (2). The patient was found to have a mutation in *CTSC*, which encodes dipeptidyl peptidase I (DPPI), a lysosomal cysteine proteinase that converts inactive precursors of granule

serine proteases into active enzymes (3). DPPI levels are especially high in human neutrophils, alveolar macrophages, and their progenitors (3). Moreover, as demonstrated by Sørensen et al., DPPI-deficient neutrophils lack elastase, cathepsin G, proteinase 3 (PR3), and neutrophil serine protease 4 (NSP4), four serine proteases normally housed within azurophil granules. Consequently, it would be predicted that the absence of these DPPI-dependent serine proteases would undermine one or more critical neutrophil activities.

The antimicrobial activity within neutrophils

As phagocytic cells, neutrophils confine ingested microbes within membrane-bound phagosomes, where two coincident responses create high concentrations of antimicrobial toxins active against a

wide variety of microbes. In response to neutrophil activation, granules fuse with nascent phagosomes, directly delivering potent antimicrobial agents to ingested microbes confined within phagosomes, with less than 2% of the total neutrophil granule content discharged extracellularly in response to physiologic stimuli (4). Azurophil granules house the heme protein myeloperoxidase (MPO), proteins with direct antimicrobial action, such as defensins and bactericidal permeability-increasing protein, and serine proteases (5). Concurrent with degranulation, the phagocyte NADPH oxidase assembles at phagosomal and plasma membranes and generates superoxide anion, which rapidly dismutates to form H₂O₂. Intraphagosomal MPO rapidly consumes H₂O₂ to oxidize chloride anion to yield the potent microbicide HOCl (6). Thus, the delivery of proteins by degranulation and the generation of oxidants by the NADPH oxidase potentiate multiple synergistic interactions that attack ingested microorganisms, compromise their integrity, and promote their eventual degradation (7).

Serine proteases and neutrophil antimicrobial action

Multiple studies have linked neutrophil elastase and cathepsin G to neutrophil-mediated antimicrobial activity. For example, MPO-mediated *in vitro* killing of *Staphylococcus aureus* or *E. coli* and microbicidal activity of purified neutrophil cationic proteins are augmented by the addition of neutrophil elastase (8). Both of these bactericidal processes depend on charge-mediated alterations of the surface of susceptible organisms (9), but not on elastolytic activity (8). Although these studies were performed with isolated enzymes in conditions very unlike the protein-rich environment of the phagosome, where competing reactions with microbe- as well as host-derived proteins occur (6), mice lacking cathepsin G, neutrophil elastase, or both exhibited enhanced susceptibility to *S. aureus* and Gram-negative enteric pathogens (9–11).

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However, MPO-dependent chemistry within phagosomes derails a simple interpretation of results from murine models. The absence of either MPO or neutrophil elastase renders mice more susceptible to fatal infection after intraperitoneal inoculation with *Klebsiella pneumoniae* (11). However, MPO oxidatively inactivates neutrophil elastase, and human neutrophils that are MPO deficient release more enzymatically active neutrophil elastase than do normal neutrophils, suggesting that loss of a single component from the neutrophil phagosome modulates the overall antimicrobial activity in situ in complicated and indirect ways.

The absence of detectable serine proteases from granules of neutrophils isolated from the PLS patient described by Sørensen et al. provides a setting to assess the extent to which neutrophil elastase and cathepsin G contribute to the antimicrobial action of neutrophils. Neutrophils isolated from this patient lack elastase, cathepsin G, PR3, and NSP4, but kill representative microbes with the same kinetics as do those isolated from a control volunteer. Furthermore, neutrophils from the PLS patients fail to generate LL-37, an antibacterial peptide produced by PR3-mediated cleavage of hCAP-18, but this deficiency has no deleterious effect on pathogen killing.

Neutrophil extracellular traps and antimicrobial action

In addition to the killing that occurs within the neutrophil phagosome, an extracellular antimicrobial response has been described whereby stimulation of purified neutrophils in vitro promotes the extrusion of nucleic acids coated with histones and granule proteins that together form complex structures called neutrophil extracellular traps (NETs) and culminate in the death of the NET-producing cell (12). Since the early reports from the Zychlinsky lab characterizing the contents and functional characteristics of NETs as a novel form of cell death, investigators have explored their production in response to a variety of physiological stimuli in vitro and in vivo using animal models (13).

As reviewed recently (14), an in vivo process called “vital NETosis” (15–17) exhibits features that differ from those of the originally described NETosis process,

which is referred to as “suicidal NETosis” (14). For example, vital NETosis requires the presence of activated platelets, occurs within minutes (as opposed to hours for suicidal NETosis), and involves budding of DNA-containing vesicles from neutrophils, without perforating the plasma membrane or compromising cell integrity (14). Although both suicidal and vital NETosis result in structures containing DNA and granule proteins, the extent to which the underlying mechanisms and functional consequences of the two processes mirror each other remains unsettled. Pending further explication of the relationship between the two phenomena, it seems prudent to distinguish in experimental systems between the two types of NETosis under study, as suggested recently (14).

NETS produced by either type of NETosis can trap microorganisms, and early studies suggested that NETs might contribute to defense against infection, although supporting evidence was indirect (18). Viable organisms can be recovered from in vitro NETs (19, 20), suggesting that suicidal NETs can immobilize bacteria, thereby limiting dissemination, but do not mediate direct antimicrobial action. NET formation in vitro requires NADPH oxidase-derived oxidants, active MPO, and neutrophil elastase to drive chromatin decondensation (21); therefore, it was posited that the failure of neutrophils from neutrophil elastase-knockout mice to generate NETs in vitro underlies the increased susceptibility of these animals to fatal *K. pneumoniae* infection (21). However, the results from Sørensen and colleagues undermine this interpretation. First, the PLS patient-derived neutrophils lack all four granule serine proteinases, including neutrophil elastase, and do not form NETs, yet these cells kill *K. pneumoniae*. The disparate killing of *K. pneumoniae* by elastase-deficient neutrophils isolated from mice compared with elastase-deficient neutrophils from humans reflects the constitutive absence of defensins from murine neutrophils, one of many species-dependent differences in neutrophil antimicrobial defenses. The absence of *both* neutrophil elastase and defensins from murine neutrophil renders them unable to kill otherwise susceptible organisms. Second, the inability of neutrophil elastase-deficient

human neutrophils to form NETs in vitro has no detectable effect on neutrophil-mediated killing of organisms. Thus, as judged by assessment of antimicrobial activity of isolated neutrophil, suicidal NETosis does not contribute to neutrophil-mediated host defense against bacteria.

Neutrophil and periodontal disease: linked by dysregulated inflammation?

Severe periodontitis afflicts patients with PLS, frequently culminating in loss of teeth, as experienced by the patient described by Sørensen and colleagues. The clinical association of periodontal disease and deficiencies in the number or function of neutrophils has long been recognized, but the underlying mechanisms remain incompletely elucidated. Although the link would seem obvious, given the predominant role of neutrophils in defense against bacteria and the presence of a robust proinflammatory microflora in the periodontal space (22), extensive data suggest that defective neutrophil-mediated antimicrobial action alone fails to explain the observed association (23). Periodontitis and premature tooth loss do not occur more frequently than normal in patients with chronic granulomatous disease (CGD), despite neutrophils from CGD patients exhibiting a profound defect in killing and the serious infectious complications seen in affected patients (24). Early onset periodontitis frequently complicates congenital neutropenias, including severe congenital neutropenia (SCN), which is commonly associated with mutations in the gene encoding neutrophil elastase (25). Periodontitis in SCN correlates with excess IL-1 β locally as well as with the oral microflora skewing toward more pathogenic organisms (26). It is conceivable that the absence of granule serine proteases and LL-37 from the neutrophils of PLS patients not only limits the array of antimicrobial effectors, but also undermines the proteolytic degradation of proinflammatory chemokines and cytokines necessary for tissue homeostasis, resulting in excess neutrophil recruitment and unchecked local inflammation (27). In such a scheme, the immunoregulatory activities of neutrophils, not their contribution to antimicrobial action per se, promote the excess inflammation and local tissue damage manifested in severe periodontitis.

Lessons learned, unknowns confirmed

The study by Sørensen et al. refines our map of how human neutrophils contribute to normal host defense. First, human neutrophils lacking serine proteases are not defective in killing ingested bacteria, and the PLS patient in this study did not have either frequent or severe infections complicating her clinical picture. Although these data refute the notion that antimicrobial actions of neutrophils require activation of granule proteases (10), as many as 25% of PLS patients experience frequent infection (28) and granule proteases very likely contribute to overall effective antimicrobial action in normal neutrophils.

Second, the paucity of infectious complications in the patient presented by Sørensen et al. suggests that an inability to support in vitro NETosis does not compromise host defense in an individual with normal neutrophil-mediated intraphagosomal killing. Because the relationship between the mechanisms and consequences of in vitro suicidal NETosis versus vital NETosis has not been elucidated, speculation about the ability of PLS neutrophils to support vital NETosis lacks sound rationale.

Third, the mechanistic links between severe periodontal disease and neutrophil dysfunction remain undefined, although the patient described by Sørensen et al. suggests that serine protease activity in neutrophils may be essential for proper neutrophil-dependent immunomodulation and maintenance of inflammatory homeostasis with the specialized microflora in the gingival crevices. Together, the findings of Sørensen and colleagues provide a revised map to guide future exploration of the way in which human neutrophils contribute to host defense against infection.

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