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Invited Perspective

Low molecular weight heparins prevent the induction of autophagy of activated neutrophils and the formation of neutrophil extracellular traps

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ABSTRACT

The protection exerted by neutrophils against invading microbes is partially mediated *via* the generation of neutrophil extracellular traps (NETs). In sterile conditions NETs are damaging species, enriched in autoantigens and endowed with the ability to damage the vessel wall and bystander tissues, to promote thrombogenesis, and to impair wound healing. To identify and reposition agents that can be used to modulate the formation of NETs is a priority in the research agenda. Low molecular weight heparins (LMWH) are currently used, mostly on an empirical basis, in conditions in which NETs play a critical role, such as pregnancy complications associated to autoimmune disease. Here we report that LMWHs induce a profound change in the ability of human neutrophils to generate NETs and to mobilize the content of the primary granules in response to unrelated inflammatory stimuli, such as IL-8, PMA and HMGB1. Autophagy consistently accompanies NET generation in our system and autophagy inhibitors, 3-MA and wortmannin, prevent NET generation. Pretreatment with LMWH *in vitro* critically jeopardizes neutrophil ability to activate autophagy, a mechanism that might contribute to neutrophil unresponsiveness. Finally, we verified that treatment of healthy volunteers with a single prophylactic dose of parnaparin abrogated the ability of neutrophils to activate autophagy and to generate NETs. Together, these results support the contention that neutrophils, and NET generation in particular, might represent a preferential target of the anti-inflammatory action of LMWH.

1. Introduction

Neutrophils are a first line of defense against infectious agents. Possibly because of the need of an effective intravascular immunity against invading microbes, neutrophils exert pro-thrombotic actions that are at least partially mediated *via* their ability to extrude decondensed chromatin bound to granular molecules. These structures are referred to as neutrophil extracellular traps (NETs) and are known to be generated *in vivo* during bacterial, fungal, protozoan and viral infection [1–3]. NETs are important for the microbicidal potential of neutrophils, in particular when they are immobilized, adherent to endothelial cells or the extracellular matrix and thus fail to phagocytose invading microorganisms [4–6]. NET generation requires the citrullination of histones by peptidylarginine deiminase 4 (PAD4), which is necessary for chromatin decondensation [7,8], and the reorganization of the neutrophil intracellular vesicles, with fusion of granules and nuclear membranes and the eventual formation of a meshwork of DNA associated to granular molecules, such as myeloperoxidase (MPO) [9]. Activation of autophagy has been previously shown to be involved, possibly both to sustain the metabolic requirements and the extensive vesicle remodelling associated to the process [10–14].

Although NET generation appears to play a critical role in host defense, it also represents a potential threat to tissue integrity in sterile inflammation, since NETs are a template on which autoantigens are assembled [2,15,16]. Also, and especially in conditions where NET generation depends on the cross-talk of neutrophils with activated platelets or platelet-derived microparticles [1,4,12,17–20], thrombogenesis may occur [19,21,22].

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Potentially damaging effects of NETs depend on the ability to trigger the generation of thrombin, the production of various cytokines and the activation of the complement cascade depending on the context in which they are generated [23–26]. In turn, inflammatory signals recruit and activate neutrophils, with further local release of NETs that results in a self-sustaining amplificatory feed-forward loop [3,16,27,28]. NET aggregation limits their potential threat to tissue integrity [29]. However, even NETs aggregates can be harmful. For example they can obstruct the lumen of biliopancreatic ducts and thus foster pancreatic inflammatory damage and remodelling [23].

Several strategies are being evaluated for their potential as regulators of NETs generation and accumulation, including the pharmacological inhibition of PAD4 [30] and the interference with the cross-talk between platelets and neutrophils [31,32].

Heparin is a mixture of multifunction glycosaminoglycans, with both antithrombin dependent and antithrombin independent anticoagulant activities. Unfractionated heparin dismantles NETs once they have been formed, releasing histones from the chromatin backbone and destabilizing the extracellular DNA lattice [13,21,25]. Conversely, circulating histones have been demonstrated to bind with high affinity to heparin even in the presence of DNA, impairing its anticoagulant action [33].

Heparins, including low molecular weight heparins (LMWH), modified derivatives of unfractionated heparin, have immune modulating actions that are independent of their anticoagulant properties [34]. The mechanisms are not yet completely clarified,

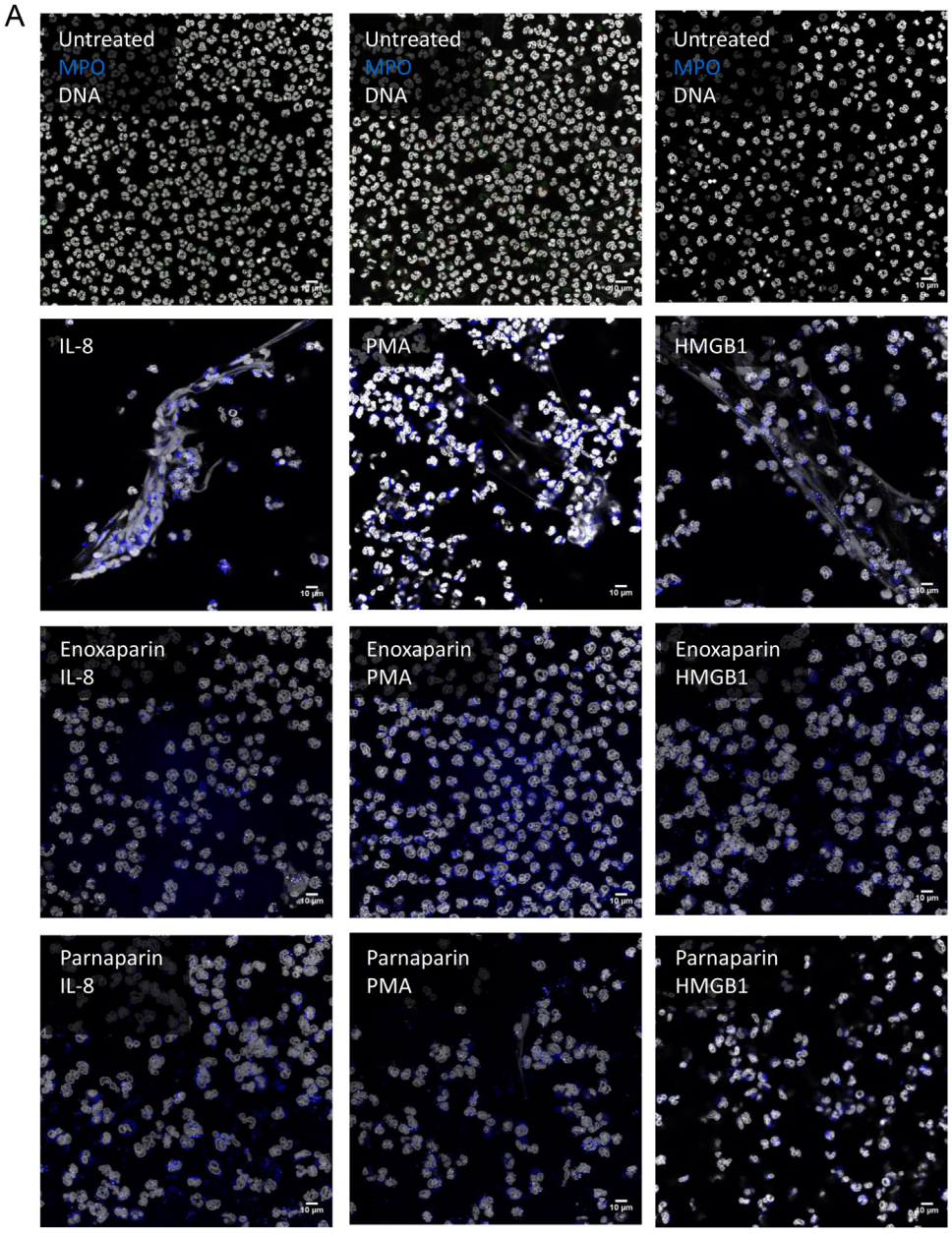

Fig. 1. LMWHs prevent the NET formation. Adherent human neutrophils, pretreated or not with enoxaparin or parnaparin were stimulated or not with IL-8 (100 ng/mL) (n = 6), PMA (100 ng/mL) (n = 3), or HMGB1 (10 μg/mL) (n = 6). A. Cells were analyzed by confocal imaging after staining with mAbs against MPO (blue color). DNA was counterstained with Hoechst (white). B. Cell-free soluble MPO-DNA complexes (OD arbitrary units, y axis) were quantified by ELISA in the supernatants of neutrophils treated or not with the relevant agonist in the presence of increasing concentrations of enoxaparin or parnaparin (anti Xa IU/mL, x axis). Background OD values observed in cell-free supernatants of untreated neutrophils was 0.1593 ± 0.0216 (mean ± SEM of eight independent experiments carried out with different donors). See Methods for experimental details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)