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Research Paper

Lactoferrin Suppresses Neutrophil Extracellular Traps Release in Inflammation

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ABSTRACT

Neutrophils are central players in the innate immune system. They generate neutrophil extracellular traps (NETs), which protect against invading pathogens but are also associated with the development of autoimmune and/or inflammatory diseases and thrombosis. Here, we report that lactoferrin, one of the components of NETs, translocated from the cytoplasm to the plasma membrane and markedly suppressed NETs release. Furthermore, exogenous lactoferrin shrunk the chromatin fibers found in released NETs, without affecting the generation of oxygen radicals, but this failed after chemical removal of the positive charge of lactoferrin, suggesting that charge-charge interactions between lactoferrin and NETs were required for this function. In a model of immune complex-induced NET formation in vivo, intravenous lactoferrin injection markedly reduced the extent of NET formation. These observations suggest that lactoferrin serves as an intrinsic inhibitor of NETs release into the circulation. Thus, lactoferrin may represent a therapeutic lead for controlling NETs release in autoimmune and/or inflammatory diseases.

1. Introduction

Neutrophil migration to inflammatory sites and subsequent phagocytosis and intracellular elimination of microbes is essential for host defense (Nathan, 2006). Activated neutrophils also release chromatin fibers called neutrophil extracellular traps (NETs), which trap and kill bacteria (Brinkmann et al., 2004), tuberculosis pathogens (Ramos-Kichik et al., 2009), fungi (Urban et al., 2009), and parasites (Guimarães-Costa et al., 2009). NETosis, the process of NET formation, includes plasma membrane rupture and chromatin fiber release following the collapse of the nuclear membrane (Fuchs et al., 2007).

While NETs are beneficial for host defense, their formation can also be harmful. For example, in sepsis (Clark et al., 2007) and transfusion-related acute lung injury (TRALI) (Caudrillier et al., 2012), NET formation induces damage to endothelial cells leading to massive thrombosis in target organs such as the lungs and kidneys. NETs may also serve as a source of auto-antigens that activate plasmacytoid dendritic cells (pDCs), thus triggering the activation of autoreactive B cells in the context of autoimmune diseases (Lande et al., 2011). In systemic lupus erythematosus (SLE), a reduction in NET degradation due to the impaired functioning of DNase1 results in the progression of lupus nephritis (Hakkim et al., 2010), and in autoimmune small-vessel vasculitis, *anti*-neutrophil cytoplasmic antibody (ANCA) directly stimulates

Abbreviations: LLf, lactoferrin; NET, neutrophil extracellular trap; MPO, myeloperoxidase; TRALI, transfusion-related acute lung injury; pDC, plasmacytoid dendritic cells; ANCA, *anti*-neutrophil cytoplasmic antibody; SLE, systemic lupus erythematosus; PMA, phorbol 12-myristate 13-acetate; GA, glutaric anhydride; SA, succinic anhydride; HOCl, hypochlorous acid; LPO, lactoperoxidase; ROS, reactive oxygen species; RPA, reverse passive Arthus.

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neutrophils to drive NET formation (Kessenbrock et al., 2009). In addition, the important role of NETs in the pathogenesis of atherosclerosis has been reported (Knight et al., 2014; Döring et al., 2014).

We previously studied the mechanism of neutrophil-dependent vasculitis (Hirahashi et al., 2006) and glomerulonephritis (Hirahashi et al., 2009) and found that activation of leucocyte β_2 integrin Mac-1 (CD11b/CD18, CR3) is a critical determinant of disease activity and inflammation-induced thrombosis. Because NET formation requires Mac-1 activation and is a downstream effector for neutrophil-mediated cytotoxicity (Neeli et al., 2009), we hypothesized that a substance that safely regulates NET formation may have applications in the treatment of inflammatory diseases.

Lactoferrin (Lf), which is found mainly in human exocrine fluids (such as maternal milk or tears) and specific granules (secondary granules) of human neutrophils (Broxmeyer et al., 1978), exhibits antibacterial properties (Pütsep et al., 2002) and possesses a strong positive charge. In addition to DNA and histones, NET fibers contain extranuclear proteins and proteins such as elastase, myeloperoxidase (MPO), and Lf, which are components of neutrophil granules (Urban et al., 2009). Although elastase and MPO are essential for NET formation (Papayannopoulos et al., 2010), the biological significance of Lf remains unclear.

In this study, we investigated the contribution of Lf to NET formation during inflammation. We developed a real-time cell imaging system using confocal fluorescence microscopy that can be used to visualize neutrophil activation, DNA and cell membrane structures by utilizing our original probes for reactive oxygen species in the cells. Here, we identified Lf as an intrinsic inhibitor of NETs release and a promising therapeutic target in pathological conditions related to NETs.

2. Materials and Methods

2.1. Isolation of Neutrophils and Platelets

Human peripheral blood samples (15 mL) were obtained from healthy volunteers who were not taking any regular medication. Neutrophils were isolated using Mono-Poly Resolving Medium (DS Pharma Biomedical, Japan) from whole blood collected in EDTA. Neutrophils were suspended in culture medium containing DMEM supplemented with 2% heat-inactivated human serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and 4 mM L-glutamine (Sigma-Aldrich) at 8 °C. Cell purity (>95%) was confirmed by Giemsa staining. Platelets were isolated at room temperature according to previously published methods (Müller et al., 2003). All procedures were conducted with permission from the medical ethics committee of The University of Tokyo, Japan.

2.2. Analysis of NET Formation

Neutrophils were seeded at 1×10^5 cells/well in μ-Slide 8-well plates (Ibidi, Germany) and incubated for 30 min with 2–200 μg/mL Lf from human neutrophils (Sigma-Aldrich) or bovine milk (Sigma-Aldrich), 200 μg/mL angiogenin, 200 μg/mL lactoperoxidase, 200 μg/mL G-Lf, 200 μg/mL S-Lf (generated as described for the charge-conversion of Lf), 10 μM diphenyleneiodonium chloride (DPI; Sigma-Aldrich) as an NADPH oxidase inhibitor, 1 mM 3-amino1,2,4,triazole (3AT; Wako, Japan) as a catalase inhibitor, 200 U/mL catalase (Sigma-Aldrich), 5 ng/mL recombinant human tumor necrosis factor alpha (TNFα; R&D Systems) and/or 200 μg/mL transferrin (Wako), and 10 U/mL heparin as a negatively charged molecule. Neutrophils were then stimulated with 12.5–25 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 3 h, 5 μg/mL monoclonal antibodies targeting MPO (MPO-ANCA, Acris Antibodies, USA, Cat# SM1475P RRID:AB_1005506) for 3 h, and 5×10^6 platelets (for 5×10^5 neutrophils) for 1 h. For live cell imaging, neutrophils were incubated with 500 nM SYTOX Green (Invitrogen/Life Technologies), 5 μM DRAQ5 (Abcam) for staining DNA, 500 nM HySOx for probing hypochlorous acid (HOCl), and/or 5 μg/mL WGA (Invitrogen/Life Technologies) for staining the cell membrane. Confocal fluorescence microscopy (Leica SP-5) was used to visualize NET formation at 37 °C. The fluorescence intensities of SYTOX Green, HySOx- and tetramethylrhodamine-labeled WGA, and DRAQ5 were determined at 500–540, 560–620, and 650–750 nm, respectively, with excitation at 490, 550, and 630 nm, respectively. For quantification of NET formation, we counted the number of cells that released SYTOX Green fluorescence into the extracellular space by reference to the report by Brinkmann et al. (2010) and divided this number by the total number of cells in the same field. Furthermore, we used Picogreen dsDNA assay reagent to quantify NET-DNA from 1×10^5 cells per 400 μL culture medium. Activated platelet-induced NET formation was evaluated as previously described (Clark et al., 2007). Briefly, 5×10^5 neutrophils were pretreated with 10 μM SQ29548 (Santa Cruz Biotechnology; a selective thromboxane receptor antagonist) for 10 min and 200 μg/mL Lf for 30 min before stimulation. Platelets were pre-activated with or without 50 μM thrombin receptor-activating peptide (TRAP, a PAR-1 agonist; Sigma-Aldrich) for 30 min and incubated with neutrophils for 1 h at 37 °C. Soluble IC-induced NETosis was evaluated as previously described (Chen et al., 2012). For preparation of soluble ICs, BSA and *anti*-BSA antibodies were mixed at 4–6 times antigen excess and incubated at 37 °C overnight as previously described (Stokol et al., 2004). For each experiment, the measured values were averaged from three replicates after the background was subtracted.

2.3. Analysis of NETs in Reverse Passive Arthus (RPA) Reaction In Vivo

Analysis of NET-like structures was performed as described previously (Chen et al., 2012). Briefly, male eight-week-old WT C57BL6/j mice were treated with 20 mg/kg bovine Lf or PBS alone, followed by administration of an intrascrotal injection of *anti*-BSA antibodies (200 μg/300 μL, Sigma-Aldrich) and an intravenous injection of BSA (300 μg/100 μL, Sigma-Aldrich) to induce RPA reaction as previously reported (Stokol et al., 2004). After 3 h, mice were injected with SYTOX Green (25 nM, Molecular Probes, USA), and the cremaster muscle was prepared for intravital microscopy. Images were obtained using an upright epifluorescence microscope (FV1000, Olympus Imaging, USA) with a 40× water-immersion objective. Images were recorded with a CCD camera (DP72, Olympus Imaging) and analyzed using ImageJ software (NIH). SYTOX Green-positive individual NET fibers were counted in 8–10 representative images (436 μm × 328 μm). Data are presented as the average number of fibers (±standard error of the mean [s.e.m.]) per mm^2. Signals from intact SYTOX Green-positive cells were excluded from this analysis, and only those exceeding 20 μm in length were considered NET-like formations. The number of neutrophils was quantified using reflected light oblique transillumination in the area 75 μm to each side of the vessel over a 100-μm vessel length (1.5×10^{-4} mm^2).

2.4. Immunofluorescence Microscopy

Neutrophils (1×10^5) were stimulated with 25 nM PMA in the presence or absence of 200 μg/mL Lf and fixed on microslide glass (Matsunami, Japan) using cytospin. Neutrophils were fixed with 4% paraformaldehyde and blocked with 3% BSA (Sigma-Aldrich) for 1 h and incubated for 1 h at room temperature with fluorescently conjugated *anti*-lactoferrin antibodies (1:100, HyTest Ltd., Finland, Cat# 4L2-2B8 RRID:AB_1618690) labeled with Alexa 488 SE (Invitrogen/Life Technologies), *anti*-elastase antibodies (1:200, Calbiochem/Merck Millipore, Germany, Cat# 481001, RRID:AB_212213) labeled with Alexa Fluor 546 (Invitrogen/Life Technologies), or DRAQ5 for staining DNA or with 5 μg/mL WGA for staining cell membranes. A fluorescence microscope (Leica SP-5) equipped with Z-axis imaging and DIC was used to collect images. We used ImageJ processing software to normalize and quantify the signals of Lf relative to those of the background.