Neutrophil serine proteases exert proteolytic activity on endothelial cells

Uwe Jerke[1], Daniel Perez Hernandez[2], Patrick Beaudette[2], Brice Korkmaz[3], Gunnar Dittmar[2] and Ralph Kettritz[1,4]

[1]Experimental and Clinical Research Center, a joint cooperation between the Charité and the Max-Delbrück Center for Molecular Medicine (MDC), Berlin, Germany; [2]MDC, Berlin, Germany; [3]INSERM U-1100 Universite Francois Rabelais, Tours, France and [4]Nephrology and Intensive Care Medicine, Charité Campus Virchow, Berlin, Germany

Neutrophil serine proteases (NSPs) are released from activated neutrophils during inflammation. Here we studied the transfer of the three major NSPs, namely proteinase 3, human neutrophil elastase, and cathepsin G, from neutrophils to endothelial cells and used an unbiased approach to identify novel endothelial NSP substrates. Enzymatically active NSPs were released from stimulated neutrophils and internalized by endothelial cells in a dose- and time-dependent manner as shown by immunoblotting, flow cytometry, and the Boc-Ala substrate assay. Using terminal-amine isotopic labeling of substrates in endothelial cells, we identified 121 peptides from 82 different proteins consisting of 36 substrates for proteinase 3, 30 for neutrophil elastase, and 28 for cathepsin G, respectively. We characterized the extended cleavage pattern and provide corresponding IceLogos. Gene ontology analysis showed significant cytoskeletal substrate enrichment and confirmed several cytoskeletal protein substrates by immunoblotting. Finally, ANCA-stimulated neutrophils released all three active NSPs into the supernatant. Supernatants increased endothelial albumin flux and disturbed the endothelial cell cytoskeletal architecture. Serine protease inhibition abrogated this effect. Longer exposure to NSPs reduced endothelial cell viability and increased apoptosis. Thus, we identified novel NSP substrates and suggest NSP inhibition as a therapeutic measure to inhibit neutrophil-mediated inflammatory vascular diseases.

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Correspondence: *Ralph Kettritz, Lindenberger Weg 80, 13125 Berlin, Germany. E-mail: kettritz@charite.de*

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Neutrophil interactions with the endothelium provide an early step in vascular inflammation, including anti-neutrophil cytoplasmic autoantibody (ANCA)–mediated small-vessel vasculitis.[1–5] ANCA, and many additional stimuli, activates neutrophils causing degranulation of granule proteins.[6] Released granule proteins comprise the neutrophil serine proteases (NSPs), namely high-abundant proteinase 3 (PR3), human neutrophil elastase (HNE), cathepsin G (CG), and the very recently described low-abundant elastase-related NSP4.[7,8] Neutrophil-released NSPs exert several effects, such as processing inflammatory mediators and extracellular matrix proteins, thrombus formation, or engaging protease-activated receptors.[9] We showed previously that active NSPs contribute to ANCA-induced vasculitis by generating mature IL-1β in phagocytes.[10] Possibly, NSPs enter the endothelial cell (EC) when neutrophils interact with the vasculature. Were enzymatic activity preserved during this process, multiple intracellular proteins would be cleaved affecting endothelial structure and function. However, only a few endothelial NSP substrates have been characterized. Candidate approaches with purified NSPs revealed that PR3 cleaves p21(Waf1/Cip1/Sdi1),[11] whereas PR3 and HNE degraded the p65 NF-κB subunit[12], both processes contributing to EC apoptosis. It is reasonable to assume that additional substrates exist. Characterization of novel NSP substrates could give additional insight into mechanisms used by activated neutrophils to damage the endothelium. Detailed information on both cleavage site and context would also help in designing synthetic protease inhibitors. Recently, terminal-amine isotopic labeling of substrates (TAILS) was introduced for proteome-wide detection of neo-N-termini that are generated during proteolytic cleavage.[13] We investigated the NSP transfer from neutrophils to endothelial cells, applied TAILS to identify novel endothelial NSP substrates, and explored the effect of NSPs released from ANCA-activated neutrophils on both EC cytoskeletal architecture and barrier function.

RESULTS

Enzymatically active PR3, HNE, and CG are released from activated neutrophils and acquired by ECs

We first assessed the NSP release from activated neutrophils, their transfer to ECs, and whether or not EC-acquired NSPs

remained enzymatically active. Neutrophils were treated with buffer, fMLF, and ionophore A23187, respectively. fMLF and ionophore A23187 provoked strong PR3, HNE, and CG release into the supernatant (Figure 1a). These cell-free supernatants contained significant serine protease activity (Figure 1b). When incubated with cell-free supernatants from activated neutrophils, ECs acquired each of the three NSPs and significant serine protease activity, as indicated by immunoblotting and Boc-Ala assay (Figure 1c and d). Flow cytometry on either live or permeabilized ECs was performed to determine whether or not NSPs had bound to the EC plasma membrane or accumulated within the cells. Suitable antibodies for flow cytometry were available for PR3 and HNE, but not for CG. We detected negligible PR3 and HNE staining on the EC surface of live cells, whereas significant intracellular NSP accumulation was found (Figure 1e). Together, these experiments demonstrate that ECs acquire proteolytically active NSPs from cell-free supernatants released by stimulated neutrophils. To gain first insight into serine protease inhibitor effects, we used cell-penetrating AEBSF[14], DFP[15], and PMSF[16], as well as cell-impermeable α1-AT, respectively. When added to fMLF-induced neutrophil supernatants, all four inhibitors significantly reduced the serine protease activity by Boc-Ala (not shown) and prevented serine protease activity acquisition by ECs (Figure 1f). α1-AT, but not AEBSF, DFP, and PMSF, abrogated the NSP transfer from the supernatant to the ECs, as shown by immunoblotting. Conversely, when we pre-incubated the ECs with the inhibitors and then added the supernatant from fMLF-stimulated neutrophils, AEBSF, DFP, and PMSF, but not cell-impermeable α1-AT, reduced serine protease activity acquisition by ECs. NSP transfer was not decreased under these conditions.

ECs acquire purified PR3, HNE, and CG in a dose- and time-dependent manner

We next used purified NSPs to study the endothelial transfer of each protease separately. After incubating ECs with PR3, HNE, or CG for 60 min, immunoblotting confirmed acquisition for PR3, HNE, and CG, respectively (Figure 2a). Moreover, enzymatic serine protease activity was recovered from the ECs after incubation with each NSP (Figure 2b). Serine protease activity was also recovered when primary human umbilical vein endothelial cells (HUVECs) were incubated with the NSPs for 60 min instead of the endothelial cell line ECV304. The Boc-Ala Vmax was 3.5 ± 1.3 with HBSS, 39.5 ± 3.4 with 10 μg/ml PR3, 64.8 ± 11.6 with 10 μg/ml HNE, and 59.3 ± 12.5 with 10 μg/ml CG ($n = 3$). For an antibody-independent NSP detection assay, we directly labeled the proteases and bovine serum albumin (BSA) as a control protein. Significant uptake was observed when ECs were incubated with Alexa488-labeled PR3, HNE, and CG (Figure 2c and d). Acquisition started between 5 and 15 min, and it increased up to 60 min (Figure 2e). By confocal microscopy, acquired HNE and CG showed a rather granular staining pattern, whereas PR3 resulted in diffuse cytoplasmic staining (Figure 2f). We titrated a dose–response curve using Alexa488-labeled purified NSPs (Figure 3a). The data indicate increased NSP acquisition over a range from 0.5 to 10 μg/ml. We selected purified PR3 for additional experiments and incubated ECs with increasing PR3 concentrations for 60 min. We assayed PR3 acquisition by immunoblotting and ELISA, for serine protease activity by the Boc-Ala assay, and for PR3-specific activity using a FRET assay, respectively (Figure 3b–e). Up to this point, our data established that enzymatically active PR3, HNE, and CG were acquired by ECs in a time- and dose-dependent manner, leading to intracellular NSP accumulation.

Identification of endothelial NSP substrates by TAILS and generation of extended cleavage signatures

Our next goal was to identify novel EC substrates and to generate extended cleavage signatures for each of the three NSPs. We used ECs rather than a peptide library and an unbiased approach by applying TAILS combined with tandem mass spectrometry. Sonicated ECs were incubated with buffer control or with PR3, HNE, and CG, respectively. We performed three independent experiments, each including two experimental duplicates. We considered peptides to be an NSP substrate when the peptide was identified in at least two independent experiments, when an arginine was found at the C terminus, and when abundance was more than 6-fold higher in the NSP sample compared with the buffer sample. This conservative approach favors a high true positive prediction rate while limiting the number of identified substrates. We identified 56 peptides from 36 endothelial proteins that were cleaved in samples treated with PR3, 34 peptides from 30 proteins with HNE, and 30 peptides from 28 proteins with CG. Twelve proteins were found in two of the three NSP-treated samples. Proteins (Table 1) and the corresponding identified peptides are listed (Supplementary Table S1 online) and a Venn diagram is shown in Figure 4a. These peptides were grouped according to gene ontology classes and showed significant enrichment for cytoskeletal and cytoskeleton-interacting proteins (Figure 4b). On the basis of the sequences of identified peptides, we determined the proteolytic signature for PR3, HNE, and CG, generated corresponding IceLogos (http://iomics.ugent.be/icelogoserver/logo.html), and provided amino acid-based heat maps on both sides of the cleavage sites from P10 to P10′ (Figure 4c). Each protease showed a distinct preference for hydrophobic amino acids at the cleavage site (P1). PR3 and HNE were enriched ($P < 0.05$) for the small hydrophobic amino acids (V, C, A) and the polar (T) amino acid at P1 position. CG accommodated preferentially hydrophobic (L and M) or aromatic (F and Y) amino acids at the P1 position and was more clearly defined before the cleavage site, with an aromatic site in P2 (H) and an acidic site (D, E) at P3, followed by an hydrophobic residue (M and A). An Asp and a Tyr were mainly found at the P2 position of PR3 and HNE substrates, respectively. A common profile of small neutral amino acids (G, A, and S) was observed for all NSPs at the P1′