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Neutrophil-derived serine proteases modulate innate immune responses

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1. ABSTRACT

The serine proteases cathepsin G, human leukocyte elastase and proteinase 3 are major contents of neutrophils and are released at sites of inflammation. Although the traditional function of neutrophil-derived antimicrobial proteases is to ingest and kill bacteria, recent studies provided evidence that these proteases are able to activate specifically pro-inflammatory cytokines and lead to the activation of different receptors. Neutrophil serine proteases might therefore be important regulators of the inflammatory innate immune response and are interesting targets for new therapeutic approaches against inflammatory disorders. This review summarizes the current knowledge on the molecular regulation of the innate immune response by neutrophil-derived serine proteases.

2. INTRODUCTION

Neutrophil infiltration is a common pathological feature in acute inflammatory disorders. The primary function of neutrophils is the phagocytosis and eradication of microorganisms. After being generated in the bone marrow the neutrophils circulate in the bloodstream. They adhere to the endothelium and migrate to the infected site where they release oxygen radicals and their internal constituents, which are stored in different granules (1). The azurophil granules, also called primary granules, contain the serine proteases human leukocyte elastase (HLE), cathepsin G (CG) and proteinase 3 (PR3) in high concentrations around 1 pg enzyme per cell (2). As azurophil granules undergo limited exocytosis in response to stimulation compared with the other neutrophil granules,

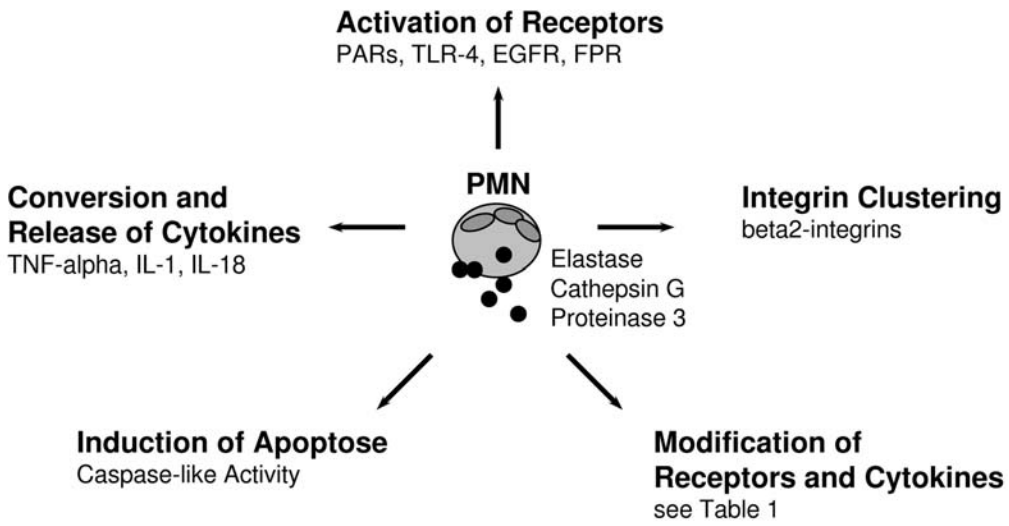

Figure 1. Different effects of neutrophil serine proteases during the innate immune response.

it is believed that their primary function is to kill and degrade the engulfed microorganisms in the phagolysosome. Considering the acidic milieu within the phagolysosome it is questionable whether intralysosomal proteolysis is the only function of these enzymes, which have an activity optimum around neutral pH values. Recently a novel mechanism has described how neutrophils release granule proteins and chromatin that together form extracellular fibers to bind and kill bacteria (3). HLE is present in these fibers and plays a crucial role in targeting and cleaving bacterial virulence factors (4).

However, neutrophils are also present in inflammatory disorders without bacterial involvement. Their active enzymes are detectable at sites of inflammation even though protease inhibitors exist in plasma and in the tissue. For example, active HLE is detectable and elastase proteolytic activity can be quantified on the surface of psoriatic plaques in psoriasis, an inflammatory skin disorder with a typical neutrophil infiltrate into the epidermis. HLE activity corresponded to the level of inflammation and disappeared after successful treatment (5).

In recent years more and more attention has focused on the extracellular effects of neutrophil serine proteases. Whilst the interest aimed in the beginning more at the deleterious potential like their matrix-degrading activity, various studies have shown evidence that neutrophil serine proteases aim specifically at a variety of regulatory functions in local inflammatory processes. Like the manifold diseases where neutrophils contribute to the pathological picture, these studies focused on different organs, cells and molecules. This review summarizes the knowledge that neutrophil serine proteases process cytokines and growth factors and stimulate various cellular receptors and that they are therefore rather an important regulatory tool in innate inflammatory processes than a nonspecific degradation machinery.. Furthermore, it will discuss that neutrophil serine proteases can act as exogenous caspase-like enzymes, are involved in integrin clustering and inactivate signaling receptors as well as cytokines selectively (Figure 1.). Knowing the molecular function of neutrophil serine proteases is necessary to understand the pathology of inflammatory disorders and may help to find new therapeutic approaches in future.

3. CONVERSION OF CYTOKINES AND GROWTH FACTORS

3.1. Proteinase 3 converts TNF-alpha

Enzymatic processing of cytokine and growth factor precursors after translation is a common principle of their activation and allows a fast response to various stimuli. One of the most important cytokines during inflammation is TNF-alpha, which was demonstrated impressively by the therapeutic success of anti-TNF-alpha treatment in Crohn's disease, rheumatoid arthritis and psoriasis. TNF-alpha is produced as a membrane- bound pro-form, which needs to be cleaved proteolytically to be released in its major biological active form. The membrane-bound metalloproteinase TNF-alpha converting enzyme (TACE) is responsible for TNF-alpha processing. However, serine protease inhibitors suppressed the secretion of TNF-alpha from activated macrophages (6;7). Moreover, mice pretreated with the serine protease inhibitor alpha1-antitrypsin were not able to secrete TNF-alpha in response to d-galactosamine together with lipopolysaccharide (LPS) thus becoming fully protected against d-galactosamine/LPS-induced hepatitis (8). PR3, but not HLE, is able to process TNF-alpha *in vitro* into its active form (9). Cleavage occurs between Val_{77} and Arg_{78} and differs from the TACE cleavage sites Val_{79} and Asp_{80} without affecting the biological activity significantly. Another report showed that HLE and CG were able to diminish the level of membrane TNF-alpha in pro-TNF-alpha-transfected TACE-negative fibroblast (10). Whether this cleavage occurs together with PR3-induced cleavage remains speculative. The cross-talk between neutrophil-derived PR3 and TNF-alpha producing macrophages was

demonstrated. PR3 from neutrophil supernatants binds to the membrane of macrophages where it contributes to the TNF-alpha cleavage and release (11).

Although anti-TNF-alpha treatment is very successful in certain diseases like Crohn's disease and rheumatoid arthritis, it runs a certain risk of promoting infectious diseases like tuberculosis. It is tempting to speculate that a more selective inhibition of TNF-alpha by PR3 inhibitors could have a benefit in theses cases as the endogenous cleavage pathway by the cell-bound TACE is not affected.

3.2. Conversion of IL-1 by proteinase 3

Another important pro-inflammatory cytokine is IL-1beta, which participates in inflammatory diseases like rheumatoid arthritis (12), neuroinflammation (13) and many others. IL-1beta, like TNF-alpha, is synthesized as an immature and inactive precursor (14;15). Conversion to the active form depends on cleavage by a cysteine proteinase, named caspase-1 or IL-1beta-converting enzyme, which also may be important for transport of the mature protein across the cytoplasmic membrane (16-18). Furthermore, the release of pro-IL-1beta from activated monocytes has also been described (19;20). Caspase-1-defcient mice can still generate mature IL-1beta in response to a local inflammatory stimulus (21) indicating that alternative processing of this cytokine *in vivo*. Activated neutrophils or purified PR3 released TNF-alpha and IL-1beta from a stimulated human monocyte cell line independent of converting enzyme release. In consideration that keratinocytes produce but are not able to process pro-IL-1beta, one may suggest a requirement for extracellular processing particularly in the context of localized inflammatory processes.

3.3. Proteinase 3 activates IL-18

Interleukin-18 is a member of the IL-1 cytokine family, which is known to be cleaved by caspase-1 to its active form. The inactive precursor of IL-18 was also identified as a substrate of PR3. The IL-18 precursor is constitutively expressed in primary human oral epithelial cells and several epithelial cell lines. When primed by IFN-gamma and subsequently stimulated with PR3 in the presence of LPS, these cells release active IL-18 into the supernatant (22). The appearance of active IL-18 was not due to cell leakage or death. The release of active IL-18 was independent of caspase-1 activity. Injection of mice with recombinant Fas ligand resulted in hepatic damage, which was IL-18-dependent. However, the same results were obtained in mice deficient in caspase-1 (23) implicating an alternative activation mode for IL-18 *in vivo*. It is quite likely that this alternative activation could be performed by proteases like PR3. Furthermore, it was reported that IL-18 is expressed by neutrophils and that its activation was mediated by the serine protease, elastase and cathepsin G (24). Neutrophils likely play a critical role in regulation IL-18 activities during early innate immune responses. Contribution of IL-18 in diseases has been demonstrated in sepsis (25), arthritis (26) and inflammatory bowl disease (27) *in vivo* models.

The processing of the pro-inflammatory cytokines TNF-alpha, IL-1b and IL-18 selectively by PR3 and not by HLE points to a crucial contribution of this protease in inflammatory processes. As PR3 is the target antigen of the c-ANCA autoantibodies in Wegener's granulomatosis, a severe inflammatory autoimmune disease, the special function of this protease is of particular interest. Besides its proteolytic modulation of cytokines it was reported that PR3 is an IL-32 binding protein, which modulates IL-32 activity by either proteolytic activation or neutralization of IL-32 (28). IL-32 is a recently discovered proinflammatory cytokine (29).

4. ACTIVATION OF RECEPTORS

4.1. Activation of PARs

The discovery of a group of seven-transmembrane receptors, which are activated directly by proteolytical cleavage and are therefore named protease-activated receptors (PARs), has dramatically changed the traditional view of protease mediated signaling. Following proteolysis, a new N-terminus of the extracellular part of PARs occurs, which now acts as a tethered ligand (30). Four members of PARs are known. PAR-1, PAR-3 and PAR-4 are activated by thrombin, and PAR-2 by trypsin and chymotrypsin. PARs are expressed in various cells and organs and the functions described so far resemble this variety. Human neutrophil serine proteases interact with PARs. CG activates PAR-4 to initiate thrombocyte aggregation beside thrombin (31). CG increased intracellular calcium in PAR-4-transfected fibroblasts, PAR-4-expressing oocytes and human platelets. A PAR-4 blocking antibody inhibited activation of platelets by CG and prevented calcium signaling in platelets. Thus CG may activate platelets and other cells at sites of injury and inflammation by cleaving PAR-4. PR3-cleaved peptide fragments of PAR-2 at the activation site and PR3-induced calcium response in oral epithelial cells were suppressed by prior desensitization of PAR-2, which suggested that PR3 is a PAR-2 activator (32). Proteolytic cleavage of PARs with loss of the tethered ligand sequence resulted in an irreversible inactivation of the receptor. HLE and CG inactivate PAR-1, PAR-2 (33), and HLE also inactivated PAR-3 (34) in the same manner. To inhibit platelet aggregation potent PAR antagonists are now under clinical evaluation suggesting therapeutic potential in thrombosis, restenosis and various inflammatory disorders (35).

4.2. HLE activates TLR4

The discovery of the family of toll receptors in insects and toll-like receptors (TLRs) in higher animals including the LPS receptor TLR-4, which is responsible for LPS-induced septic reactions, was a breakthrough in understanding and appreciating the innate immune system as a fundamental part of immunity in all living creatures. Human leukocyte elastase up-regulates IL-8 via TLR-4. A TLR-4 neutralizing antibody inhibited the HLE-induced IL-8 production from cultured cells (36). In this study HLE resulted in a decrease of TLR-4 surface expression-induced tolerance to LPS in subsequent LPS stimulations after HLE application. The same group reported previously that HLE

induces IL-8 expression in bronchial epithelial cells involving the TLR signal transducers IRAK, MyD88 and TRAF-6 (37). A possible molecular explanation was given by a study, where intraperitonally injected pancreatic elastase led to death of mice in a TLR-4 dependent manner involving the mobilization of heparan sulphate. Intraperitonally injected heparan sulphate resulted in 80% death in wild-type mice but no death in TLR-4-defcient mice (38). Injection of pancreatic elastase resulted in 50% death in wild-type animals by its proteolytic activity and no death in TLR-4-defcient mice. The authors demonstrated that injected elastase resulted in loss of heparan sulphate from blood vessels at the injection site within 5 h of injection and conclude that activation of TLR-4 by elastase-dependent release of heparan sulphate (39;40) might be an endogenous pathway to systemic inflammatory response syndrome-like reactions. However, this was only shown for pancreatic elastase and not for HLE.

4.3. HLE activates EGFR

Human leukocyte elastase has been implicated in the activation of the epidermal growth factor receptor (EGFR). HLE application on cultured keratinocytes leads to cell proliferation via EGFR involving the release of TGF-a by its proteolytic activity (41). Application of HLE on murine skin resulted in a twofold increase of epidermal layers within 3 days without a visible inflammatory infiltrate in contrast to trypsin-induced epidermal hyperproliferation (42;43). Trypsin is another serine protease with different cleavage preference than HLE. Concentrations of HLE used in these studies to induce proliferation of keratinocytes *in vitro* and *in vivo* are in the same range as concentrations found on the surface of psoriatic lesions (44). The elastase activity of psoriatic lesions correlates with the level of disease and disappears after successful treatment (5). The authors conclude that HLE is an important and relevant stimulus for epidermal proliferation in psoriasis. Using a different methodological approach it was shown that HLE leads to the induction and release of mucin in a cultured mucoepidermal cell line by proteolytical cleavage of membrane-bound TGF-a and activation of EGFR (44). It was proposed that this activation is TACE dependent (45). More studies have proven thereafter the importance of HLE-induced EGFR activation (46-49).

Induction of mucin by the activation of EGFR may be the major cause for hypersecretion in the lung (50). Chronic HLE exposure to murine lungs resulted in mucus cell hyperplasia mucin induction (51). A key role for HLE in airway secretion has been proposed for quite some time and the recent *in vivo* and *in vitro* data suggest that HLE may cause airway hypersecretion as in chronic obstructive pulmonary disease or in cystic fibrosis, where high levels of HLE are present.

4.4. Cathepsin G activates the formyl peptide receptor

It was shown that CG is a chemotactic agonist for the G-protein-coupled formyl peptide receptor (FPR) (52). CG binds FPR with low affinity, inducing a modest Ca+ flux and weak activation of mitogen-activated protein (MAP) kinases. However, the stimulation with CG of cells that express FPR induced the translocation of protein kinase C-zeta from the cytoplasm to the plasma membrane, which is essential for FPR to mediate the chemotactic activity of CG. Therefore, extracellular CG might contribute to leukocyte recruitment *in vivo* by binding to FPR on neutrophils and monocytes (53). The agonistic activity of CG on FPR might not depend on proteolysis, as no new small and soluble molecules were released during the interaction of the enzyme with the receptor. CG is a host-derived chemotactic agonist for FPR. This observation expands the functional scope of this receptor in inflammatory and immune responses.

5. CASPASE-LIKE ACTIVITY

Proteinase 3 is not just active when released into the extracellular space or in a membrane bound form, but also traverses the endothelial plasma membrane and induces apoptosis (54). The mechanism of PR3 internalization is not known, but was cell type specific as lung epithelial cells did not internalize PR3. The authors hypothesize that PR3 internalization is receptor-dependent. Exposure of endothelial cells to PR3 results in cleavage and inactivation of the transcription factor NF-kappaB and sustained activation of JNK (55). Inhibition of caspases did not block the cleavage of p65 NF-kappaB and sequence analysis exhibited that the PR3 cleavage site was unique with respect to reported caspase cleavage sites. Recently the same group demonstrated that PR3 sidesteps caspases and cleaves the cell cycle inhibitor p21 to induce endothelial cell apoptosis. PR3 cleaves p21 at Thr_{80} and Gly_81, a region susceptible to caspase-3 cleavage, although PR3 cleavage of p21 is caspase-independent demonstrated by using a broad-spectrum caspase inhibitor. Cleavage results in loss-of-function of p21 with nuclear exclusion and activation of apoptosis (56). The authors speculate that the kinetics of apoptotic activation would be accelerated because activation of the caspase cascade is not required for p21 cleavage. Subsequent phagocytosis of apoptotic cell would aid in the resolution of inflammation, as both the injured endothelial cells and PR3 would be removed from the site. It can be assumed that immune cells like neutrophils have evolutionarily acquired the capability to intervene into intracellular caspase cascades through released proteases like PR3 to combat foreign microbes that override normal apoptotic signals. In auto-inflammatory disorders like Wegener's disease, which is characterized by the appearance of auto-PR-3 antibodies and systemic vasculitis, PR3-induced endothelial apoptosis is an exciting new hypothesis to explain the common picture of vasculitis. Neutrophils themselves seem to be protected from PR3 induced procaspase-3 activated apoptosis by compartmentalized PR3-induced capase-3 activation (57). The activated 22-kDA fragment was not present when neutrophil were treated with serine proteinase inhibitors. The 22-kDa caspase-3 fragment was restricted to the plasma membrane compartment. Double immunofluorescence labeling after streptolysin-O permeabilization showed that PR3 and procaspase-3 could co-localize in an extragranular compartment. Induction of okadaic acid induced apoptosis in human neutrophils was demonstrated to be serine protease-dependent suggesting a

Table 1. Overview of different targets of neutrophil serine proteases

Target	Cleaved by	Hypothetical biological function	Reference
Receptors			
PAR-1	HLE, CG	Inactivation, modulation of response	(33)
PAR-2	HLE, CG	Inactivation, modulation of response	(33)
PAR-3	HLE	Inactivation, modulation of response	(34)
IL-2Ra	HLE, PR3	Inhibiting cellular response and prolongation of cytokine half-life time	(63)
TNF-RII	HLE	Inhibiting cellular response and prolongation of cytokine half-life time	(64)
IL-6R	CG	Inhibiting cellular response and prolongation of cytokine half-life time	(63)
C5aR (CD88)	HLE, CG	Inhibition of chemotaxis, feedback mechanism	(65)
C3b/C4b R (CD35)	HLE	Inhibition of complement signaling	(66)
Urokinase R (CD87)	HLE, CG	Regulation of cell migration	(67)
GCSF-R	HLE	Growth inhibition	(68)
Sialophorin (CD43)	HLE	Regulation of adhesion	(68)
CD16 (low-affinity IgG Fc receptor)	HLE	Impairment of natural killer cells	(69)
CD14	HLE	Inhibition of LPS-mediated cell activation	(70)
CD2, CD4 and CD8	HLE, CG	Impairment of T lymphocytes	(71)
Cytokines/chemokines			
IL-6	CG	Regulation of cytokine half-life time	(72)
TNF-alpha	HLE, CG	Regulation of cytokine half-life time	(73)
IL-2	HLE	Regulation of cytokine half-life time	(74)
IL-8	PR3	Increased chemotaxis	(75)
ENA-78	CG	Increased chemotaxis	(76)
GCSF	HLE	Growth inhibition	(68)
RANTES	CG	Chemokine modulation, HIV protection	(77)
Integrins/others			
Intercellular adhesion molecule-1	HLE, CG	Regulation of adhesion	(78;79)
Vascular endothelium cadherin	HLE, CG	Regulation of adhesion	(80)
Proepithelin	HLE	Wound healing	(81)
TGF-beta binding protein	HLE, CG, PR3	Enhanced growth factor availability	(82)
IGF-binding protein	HLE, CG	Enhanced growth factor availability	(83)

functional role in serine protease dependent mechanism of controlling apoptosis in neutrophils (58). Induction of apoptosis by all neutrophil serine proteases was also observed in human airway smooth muscle cells. Airway smooth muscle cells showed nuclear condensation and fragmentation, annexin V binding and cleaved caspase-3 expression after incubation with neutrophils as wells as purified proteases (59).

6. INTEGRIN CLUSTERING

Previous studies have shown that activation of beta2-integrins on the surface of neutrophils by crosslinking leads to intracellular signaling and cytoskeletal rearrangement (60). Raptis *et al.* have shown that *in vitro* immune-complex-activated neutrophils that lacked both neutrophil elastase and CG adhered normally to the immune-complex-coated surfaces but failed to undergo cytoskeletal reorganization (61). This defect in cytoskeletal reorganization was accompanied by decreased phosphorylation of the GTPase RAC1, which is a key regulator of the actin cytoskeleton. As a result, serine-protease-deficient neutrophils have a severe defect in chemokine release that can be reversed by the addition of catalytically active exogenous CG. These results indicate that cell-surface-bound serine proteases might modulate the activation of beta2-integrins through proteolytic cleavage. Indeed, neutrophil elastase and CG have been shown to increase the affinity of beta3-integrins expressed on the surface of platelets through limited proteolysis of the C terminus of the alphaIIb-integrin subunit (62). There is no evidence so far that neutrophil serine proteases directly activate beta2-integrins through proteolysis. Therefore, the mechanism by which CG modulates the clustering of integrins expressed on the surface of neutrophils remains elusive.

7. PROTEOLYTIC MODIFACTION OF RECEPTORS AND CYTOKINES

Many biological molecules like cytokines and their receptors contain putative cleavage sites for neutrophil serine proteases. It is therefore not surprising that many receptors, cytokines and other molecules have been found to be natural substrates for neutrophil serine proteases (Table 1). Cleavage of receptors of ligand-binding cytokine receptor ectodomains makes the cells insensitive to cytokines. In addition, cleaved receptors may be able to bind their ligands, which prolongs on the one hand their lifetime, but prevents on the other hand cytokine binding. Proteolytic cleavage of cytokines like TNF-alpha and IL-6 is often accompanied with a loss of function and might therefore be an important mechanism for down-regulating of their inflammatory response. Considering that neutrophils themselves release some of these cytokines like IL-6 or process some of these cytokines like TNF-alpha and IL-1beta, the inactivation of these cytokines might represent a direct feedback mechanism. Proteolytical cleavage of cytokines does not necessarily need to result in a loss-of-function. Truncation of some chemokines like IL-

8 and ENA-78 by PR3 and CG, respectively, exhibits a more active form of the chemokines. A weak point of the numerous reports is that these studies are mainly done *in vitro* focusing on a limited number of particular molecules. This cannot resemble the overall effect at the *in vivo* site. Especially, the chronology of releasing and inactivation of cytokines and their receptors might be the decisive factor of a proteolytical control in inflammation. The dynamic state of activation of receptors and release of cytokines by neutrophil serine proteases, on the one hand, and inactivation of cytokines, on the other, should be considered when cytokine concentrations are analyzed at sites of inflammation where high concentrations of active neutrophil proteases are present.

8. PERSPECTIVE

During the last decades evidence has pointed at other functions of neutrophil-derived serine proteases than the classically held view that they are mainly degradative enzymes. Many studies have demonstrated that neutrophil serine proteases play an important role in cell signaling and contribute to the control of the inflammatory process. Their general processing of different local precursors to active cytokines allows a fast and specific local immune response depending on what kind and how much of the precursors are expressed. They should be nowadays considered as specific regulatory tools during the innate immune response.

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Abbreviations: TNF: tumor necrosis factor, IFN: interferon, HLE: human leukocyte elastase, PR3: proteinase 3, CG: cathepsin G, TLR: toll-like receptor, IL: interleukin, PAR: proteinase activated receptor, EGFR: epidermal growth factor receptor, TACE: TNF-alpha converting enzyme

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