Neutrophil serine proteases mediate inflammatory cell recruitment by glomerular endothelium and progression towards dysfunction

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Abstract

Background. Neutrophil recruitment into glomerular tissues and reduced capillary wall integrity has been implicated in the development of vasculitic glomerulonephritis (VGN). This study investigated the stages and mechanisms through which neutrophil serine proteases (SPs), proteinase 3 (PR3) or elastase contribute to endothelial dysfunction.

Methods. Protease-induced damage to endothelium and adhesion molecule upregulation was measured by viability assays and ELISA. Neutrophil/platelet adhesion to human glomerular and umbilical vein endothelium was assessed using in vitro adhesion assays.

Results. PR3 and elastase (1 µg/mL, 2 h) significantly induced neutrophil adhesion to endothelial cells (EnC) whilst PR3 also enhanced platelet–EnC interactions. This neutrophil adhesion was associated with enhanced P-selectin expression and required CXCL8 receptor involvement, and could be inhibited by blocking the P-selectin ligand PSGL-1. SPs induced damage in a time- and dose-dependent fashion, decreasing cell monolayer integrity followed by cell membrane integrity, inducing caspase-3 activation and p21 cleavage. However, SPs caused significant EnC damage with increasing concentrations and prolonged exposures.

Conclusion. Neutrophil SPs induce a pro-adhesive phenotype in glomerular endothelium primarily by inducing neutrophil and platelet adhesion that transits to dysfunction after high/prolonged exposures. Dysregulated release of these enzymes within glomeruli may contribute to injury during diseases such as VGN.

Keywords: adhesion; elastase; neutrophils; proteinase 3; platelets

Introduction

The early stages of vasculitic glomerulonephritis (VGN) are associated with neutrophil recruitment into glomeruli, decreased capillary wall integrity and platelet accumulation. Dysregulated neutrophil serine protease (SP) release has been implicated as a mediator of disease, possibly through interactions with glomerular endothelial cells (GEnC). Proteinase 3 (PR3) and myeloperoxidase and, less frequently, elastase are targets of anti-neutrophil cytoplasm auto-antibodies (ANCA) associated with the vasculitic diseases, which can cause VGN [1–4]. During active vasculitis, there are increased plasma levels of PR3 and elastase, in complex with their natural inhibitors [5, 6]. The infusion of neutrophil elastase through renal arteries leads to localization of the enzyme on glomerular capillaries and transient renal impairment [7]. At the cellular level, neutrophil activation can lead to the formation of neutrophil extracellular traps that contain SP, myeloperoxidase and chromatin [8]. PR3 and elastase containing traps have been detected in affected human glomeruli [9] leading to the specific cleavage of soluble [10] cell surface [11] or intracellular proteins [12]. Inefficient dismantling of these traps may result in renal damage [13].

As well as stored within the neutrophil granules, PR3 is also detectable on the surface of neutrophils and is increased during periods of active vasculitic disease [14]. Neutrophil priming, by cytokines including tumour necrosis factor-α (TNFα), increases the surface expression of PR3 [14] and triggers a small release of PR3 extracellularly [15]. Even in the presence of its inhibitor, alpha-1 anti-trypsin (α1-AT), PR3 remains on the neutrophil surface and can bind ANCA, resulting in neutrophil activation and release of neutrophil granule contents, with higher levels of membrane PR3 associated with greater responsiveness to PR3-ANCA stimulation [16]. As ANCA IgG isolated from vasculitic patients is known to both enhance protease release [8] and neutrophil–endothelial interactions in vitro [17], this brings the neutrophils and their products into close association with the vascular endothelium [8].

Although EnC do not express PR3 [18–20], this protease can bind to extracellular matrix and to EnC surfaces

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Both PR3 and elastase can degrade extracellular matrix, ultimately leading to EnC detachment [21]. PR3 bound to the endothelial surface may also be internalized and detected in the cytoplasm and in the nucleus [22]. Internalization of proteolytic-inactivated PR3 can result in EnC apoptosis, which occurs after a prolonged period (24 h) [23].

A few studies have suggested that, under certain conditions, SP may activate EnC, inducing upregulation of leucocyte adhesion molecules intercellular molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [19], or triggering the release of leucocyte chemoattractant chemokines such as interleukin (IL)-8 or chemokine (C-C motif) ligand 2 [19, 20]. These studies demonstrated that the effects of PR3 and elastase were associated with new EnC transcription and occurred over periods of 24 h or longer, with the effects of PR3 independent of its proteolytic activity, when using relatively high SP concentrations, for example, up to 20 µg/mL PR3 in the presence of serum [20]. Studies undertaken in serum-free conditions have used concentrations of 2–5 µg/mL of PR3 and elastase to avoid cell detachment [10, 24, 25].

Despite elastase and PR3 sharing high-sequence homology [26], their catalytic behaviour differs. They can cleave the same substrate at different sites [27] and can be selectively inhibited by specific SP inhibitors [26]. These differences may result in different biological activity. The importance of neutrophil SP in mediating GEnC activation or injury during the development of VGN has not been fully defined. Defining this role is relevant as SP are potentially amenable to therapeutic manipulation.

The aim of this study was to determine how PR3 and elastase can trigger a series of GEnC responses and to identify the adhesion molecule/s and chemokine/s involved in this process. We also intended to elucidate the conditions which culminate in cellular damage and in a loss of endothelial layer integrity.

Methods

EnC culture and treatment
Conditionally immortalized GEnC (a gift from Dr S. Satchell) [28] and human umbilical vein endothelial cells (HUVEC) used as a comparator (obtained with informed consent from Birmingham Women’s Hospital) were grown to confluence and were treated with 1 µg/mL (unless otherwise stated) of either PR3 (Athens Research & Technology Inc., USA) or elastase (Calbiochem, UK) for 2 and 24 h or with 100 units/mL (2.5 ng/mL) TNF-α (Calbiochem, UK) [19, 20]. A bolus of neutrophils (1 × 10⁷ cells/mL of PBSA) was then perfused over the EnC surface for 4 min and the number of adhering neutrophils was recorded, as previously described [31].

EnC viability assays
Following treatment with SP, cell viability was assessed using modified tetrazolium assay for mitochondrial activity [29]. Cell membrane integrity was detected by incubating EnC with 20 µg/mL propidium iodide (Sigma) for 20 min. Cells were photographed under fluorescence microscopy (Olympus) and were counted using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). EnC apoptosis was detected using 1 µg/mL fluorescent DNA-specific dye, 4′,6-diamidino-2-phenylindole (DAPI; Sigma), by measurement of adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio using the ApoGlow® Assay (Lonza, UK) and by western blotting detecting caspase-3 activation using polyclonal rabbit anti-caspase-3 primary antibody (Invitrogen) and p21 cleavage using mouse anti-p21 Cip/WAF1 (Invitrogen).

Static EnC-Platelet adhesion assay

Washed platelets (2 × 10⁸ cells/mL) isolated from healthy volunteers were fluorescently labelled with calcine-AM (1 µg/mL; Molecular Probes, Eugene, OR, USA) for 20 min. Platelet adhesion to EnC was visualized by fluorescent microscopy and the percentage of endothelial surface covered in fluorescent platelets (% coverage) [30] was quantified using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Flow-based EnC-neutrophil adhesion assay

Neutrophil adhesion to EnC under static conditions was assessed as previously described [32–34]. For PSGL-1, CXCR1 and CXCR2 blocking studies, isolated neutrophils were pre-incubated for 15 min with 10 µg/mL anti-CD162/PSGL-1 (clone PL1 from antibodies-online GmBH, Germany), 4 µg/mL anti-CXCR1 (clone 501 from Invitrogen, UK), 4 µg/mL anti-CXCR2 (clone 19 from Invitrogen, UK) or a corresponding isotype control antibody.

EnC adhesion molecule expression by cell-based enzyme-linked immune absorbance assay

Adhesion molecule expression was assessed by ELISA using primary mouse anti-human antibodies (200 µg/mL) against E-Selectin IgG1, clone BBIG-E4 (5D11); anti-VCAM-1 IgG1, clone BBIG-V1 (4DB); IgG2a isotype control, clone 20102.1; all from R&D Systems, UK; anti-P-selectin IgG1; anti-ICAM-1 IgG2a, clone MEM-111; from AbCam, UK or isotype control IgG1 from mouse myeloma, clone MOPC21 (Sigma-Aldrich). Goat anti-mouse HRP conjugate (200 µg/mL, Dako) was the secondary antibody. Cells were washed and TMB substrate was added (Calbiochem, USA). ELISA reactions were stopped using 2 M HCl (BDH, Lutterworth, UK) and absorbance was read at 450 nm (Biotek EL808).

Statistics

ANOVA with Bonferroni or Dunnett post-tests and non-parametric Mann–Whitney were used to compare samples and cell types and P-values of <0.05 were considered to be statistically significant.

Results

Neutrophil SP regulate GEnC damage in a time- and concentration-dependent manner

Initially, the viability of GEnC was assessed by exposure to a range of PR3 and elastase concentrations under serum-free conditions to define which conditions either regulate endothelial activation without loss in viability or detrimentally affect endothelial survival.

After 2 h, there was no significant loss in viability of GEnC treated with 0.5–10 µg/mL PR3 or elastase when compared with untreated controls. The mitochondrial activity of GEnC assessed by tetrazolium assay showed no significant alterations after 2 h treatment with 0.5–10 µg/mL PR3 or elastase (Figure 1A). HUVEC displayed a similar response with PR3 treatment, but the viability of HUVEC was significantly reduced by treatment with elastase 10 µg/mL (Supplementary material Figure 2A). These results were confirmed by crystal violet assay, which was used to enumerate the percentage of adherent
viable cells following treatments (Supplementary material Figure 2B and C). Following 2 h treatment with concentrations of PR3 or elastase up to 2 µg/mL did not alter EnC monolayer integrity (morphology), membrane integrity (propidium iodide staining) and nuclear structure (DAPI staining) (Figure 1B). PR3 or elastase (1 µg/mL, 2 h) did not alter GEnC morphology or monolayer integrity (phase contrast microscopy), membrane integrity (propidium iodide staining) and nuclear structure (DAPI staining) (B). SP (1 µg/mL, 2 h) treatment did not cause caspase-3 activation in GEnC (C). Positive control is TNFα/cycloheximide (representative images from three experiments). PR3 or elastase (5 µg/mL, 2 h) mediated p21 cleavage in GEnC from pooled attached and detached cells which was not observed with SP (1 µg/mL, 2 h) treatment (D). However, with prolonged, 24 h protease treatment, viability of GEnC was significantly reduced by the presence of PR3 10 µg/mL and elastase significantly reduced viability of GEnC at concentrations as low as 2 µg/mL PR3 (E).

There was a dose-dependent cleavage of the stress marker, intracellular p21 in the absence of caspase-3 activation by western blotting (Figure 1C). There was a dose-dependent cleavage of the stress marker, intracellular p21 in the absence of caspase-3 activation by western blotting (Figure 1C). There was a dose-dependent cleavage of the stress marker, intracellular p21 in the absence of caspase-3 activation by western blotting (Figure 1C). There was a dose-dependent cleavage of the stress marker, intracellular p21 in the absence of caspase-3 activation by western blotting (Figure 1C).
under flow conditions with HUVEC, PR3 and, to a much lesser extent; elastase appeared to increase EnC–neutrophil interactions and was inhibitable by α1-AT (Figure 2D).

**SP triggered surface P-selectin expression**

At the concentrations of SP where there was increased neutrophil adhesion, 1 µg/mL PR3 significantly increased surface expression of P-selectin, on both GEnC (Figure 3A) and HUVEC (Figure 3B). Alternatively, treatment with 1 µg/mL elastase did not significantly increase in surface expression of P-selectin in both GEnC and HUVEC, whereas both cell types treated with elastase showed a dose-dependent increase in P-selectin expression with increasing concentrations of enzyme (0.5–2 µg/mL) (Figure 3A and B). Neither PR3, nor elastase, altered E-Selectin, ICAM-1 or VCAM-1 expression (receptors which require de-novo synthesis) in either GEnC or HUVEC (n = 3, data not shown).

**Blocking PSGL-1 inhibits P-selectin-mediated neutrophil adhesion**

The enhanced P-Selectin expression had a direct effect on adhesion. Addition of a blocking antibody directed at the P-selectin ligand, PSGL-1, to unstimulated neutrophils
completely abolished increases in neutrophil adhesion to GEnC (Figure 3C) or HUVEC (Figure 3D) caused by SP (1 μg/mL), whilst no inhibition occurred with addition of an isotype control.

Protease-induced neutrophil adhesion requires an ELR chemokine signal

Neutrophil adhesion to PR3 or elastase treated GEnC was abolished with blocking antibodies directed at the ELR chemokine family receptors, CXCR1, CXCR2 or both. Similar findings were observed with HUVEC (Figure 4A and B).

PR3 also enhances platelet–EnC interactions.

After observing the pro-adhesive properties of PR3 on EnC–neutrophil adhesion under static and flow conditions, the influence of PR3 on the ability of EnCs to support platelet adhesion was assessed under static conditions. PR3 treatment of EnC (1 μg/mL, 2 h) induced a small, variable, but detectable increase in platelet adhesion. Representative fluorescent (Figure 5A) or phase contrast (Figure 5B) images from treated GEnC and percentage coverage data from treated HUVEC (Figure 5C).

Discussion

ANCA-induced neutrophil-dependent inflammation leads to EnC injury via the release of proteolytic SP, as well as oxygen radicals, and by neutralization of the protective antiproteinase shield at inflammatory sites [35–37]. The
initiation of vascular injury begins with activation of the endothelium by cytokine stimulated neutrophils, which supports local leucocyte infiltration and damage [38–40]. Here, we have demonstrated differential effects of PR3 and elastase on endothelial viability and adhesiveness and clearly defined a time- and dose-dependent protease-induced transition of normal anti-inflammatory EnCs to a pro-adhesive phenotype, eventually resulting in damage. We have confirmed that high levels of both proteases can induce cell retraction and detachment [11].

Our study demonstrates that lower concentrations of SP with shorter incubation periods do not cause any significant damage to the endothelium; instead they induced a weak adhesive phenotype, which permitted neutrophil capture. The endothelial neutrophil interactions were supported by mobilizing endothelial P-selectin expression and could be stabilized by IL-8 signalling via interactions with CXC chemokine receptors. Following SP treatment to EnC, we did not detect IL-8 in supernatants. However, we could demonstrate rapid cleavage of IL-8, from TNF-α activated EnC, by these proteases (data not shown) and by previous studies by Padrones et al. [41]. The involvement of P-selectin may explain the early and transient nature of PR3 or elastase-mediated neutrophil adhesion since initial EnC stimulation does not require de novo protein synthesis, as described by Hunt and Jurd [42]. This would be compatible with the likely mobilization of Weibel–Palade bodies to the EnC surface by SP, resulting in the release of P-selectin and von Willebrand factor (vWF) (our unpublished data) within 2 h. The recruitment of neutrophils by PR3 under flow conditions may require a threshold endothelial P-selectin level, as neutrophil adhesion only occurred under conditions which substantially increased P-selectin expression; neutrophil adhesion was not discernable with elastase where P-selectin expression was not significantly upregulated. Furthermore, elastase treatment of EnC cultured on microslides caused cellular stress that was not observed with PR3, and may have contributed to the differences in neutrophil recruitment following the protease treatments under physiological flow conditions. Inducible P-selectin expression and adhesion under flow and static conditions are functional markers that highlight a difference in the responsiveness of HUVEC and glomerular cells to PR3 and elastase.

This is the first study to demonstrate a role for PR3-induced enhanced platelet adhesion to EnC. The observed platelet adhesion suggests a prothrombotic change in EnC. Protease-induced pro-thrombotic endothelial activation had previously been demonstrated [43], with increased cleavage of thrombomodulin under serum-free conditions [11]. Further, tissue factor expression was induced on HUVEC after stimulation by PR3 or elastase using serum-free conditions [43]. It has been suggested that the PR3 induction of EnC tissue factor mRNA may

Fig. 5. PR3 but not elastase (1 µg/mL) treatment significantly induced EnC–platelet interactions. Images are representative fluorescent or phase-contrast photomicrographs of platelets adhering to GEnC. The percentage coverage of adherent platelets on HUVEC monolayers were calculated (n = 3–5). Stats: one-tailed paired t-test.
occur via cleavage and activation of protease activated receptor-1 (PAR-1), although this does not support the proteolytic independent effect of PR3 in the induction of tissue factor demonstrated in the studies of Haubitz et al. [43] and other findings that PAR-1 is disarmed not activated by PR3 (our submitted data).

PR3 has previously been reported to induce CXCL-8 and monocyte chemoattractant protein-1 (MCP-1) release and ICAM-1 expression on HUVEC with prolonged exposure times (24–72 h using up to 20 μg/mL) [44, 45]. It is interesting that the increased adhesion demonstrated in these studies occurred at concentrations and at time points that have been previously shown, and confirmed here, to be associated with cell detachment and enhanced apoptosis, albeit under serum-free conditions [11, 21, 22] suggesting that in the presence of serum-derived inhibitors, these proteases may still enhance adhesion over a longer time frame via transcription of chemokines and adhesion molecule proteins.

PR3 and elastase can induce activation of pro-apoptotic signalling events through NFκB and JNK, ERK, and p38 MAPK coincided by their entry into EnC [12]. PR3 cleaves p65 NF-κB in the N-terminal region and elastase cleaves in the C-terminal region [12]. Apoptosis of EnC may also occur when these normally adherent cells detach from their extracellular matrix, a process called anoikis [11, 21, 22] although cells remaining adherent appear to maintain viability. In this present study, we were able to consider development of apoptosis in both detached and adherent cells. We observed no caspase-3 activation in protease-treated adherent cells or in samples containing both adherent and any detached cells. However, PR3 or elastase-induced p21 degradation was detectable after exposure to 5 μg/mL SP, confirming earlier studies performed with PR3 [46]. p21 can act as a regulator of both cell-cycle progression and apoptosis [47, 48] which suggests that p21 degradation prior to any detectable caspase activation may be an early indicator of protease-induced endothelial stress.

The data presented here supports previous evidence that the effects of PR3 and elastase differ both quantitatively and qualitatively. At higher concentrations and over prolonged periods, both proteases induced EnC retraction, with the effects of elastase occurring more rapidly, with lower concentrations also inducing cell damage, whilst PR3 more effectively increases P-selectin expression. It is possible that the higher endothelial injury observed by elastase may, in part, be attributed to differences in the activity of the two proteases, although one must also consider their different mechanism of actions and preferred targets [26, 27]. As expected, EnC responses differed depending on whether they were derived from large conduit vessels or capillary beds. Both SP can induce endothelial damage, but the extent to which this reflects internalization and cleavage of intracellular mediators versus induction of anoikis remains uncertain.

Altogether, these data point to a complex pattern of regulation, depending on local concentrations of SP and their inhibitors. There are three potential routes for targeting these neutrophil SP: (i) the use of specific anti-protease inhibitors [49–51] (ii) reducing protease activation by dipeptidyl peptidase I, or (iii) reducing neutrophil accumulation with phosphodiesterase inhibitors [52]. In addition, other proteins such as IL-32 [53] may bind to PR3 and influence its activatory properties. Nonetheless, regulating the effects of these enzymes in vasculitis may be a key target for therapy. Furthermore, specific targeting of neutrophil SP may be beneficial in other chronic inflammatory diseases in which neutrophils drive endothelial injury.

**Supplementary data**

Supplementary data are available online at http://ndt.oxfordjournals.org.

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