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Proteolysis of classic anti-neutrophil cytoplasmic autoantibodies (C-ANCA) by neutrophil proteinase 3

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SUMMARY

C-ANCA, which are directed against neutrophil proteinase 3 (PR3), are specific markers for the diagnosis of active Wegener's granulomatosis (WG). The correlation between C-ANCA titre and WG disease activity suggests that these autoantibodies are involved in the development of WG. We have previously observed that C-ANCA interfere with PR3 proteolytic activity and with complexation of PR3 with its major physiologic inhibitor α_1-antitrypsin (α_1-AT). The possible pathogenic importance of C-ANCA may be related to the stability of C-ANCA IgG–PR3 complexes. In the present study we tested proteolysis by PR3 of human IgG and proteolysis of C-ANCA IgG complexed to the enzyme. All human IgG subclass proteins were cleaved by PR3. Digestion products were compared with those obtained by human neutrophil elastase (HNE)-mediated proteolysis of human IgG subclass proteins. Although cleavage products of similar size could be identified, the proteolytic activity of both enzymes towards human IgG differed. Furthermore, inhibiting C-ANCA IgG were cleaved into small peptides when complexed to PR3. The possible pathogenic consequences of these findings will be discussed.

Keywords proteinase 3 human IgG C-ANCA proteolysis

INTRODUCTION

Proteinase 3 (PR3) is a neutral serine proteinase and a major constituent of azurophil granules of human neutrophils [1,2]. Upon inflammatory stimulation and subsequent neutrophil degranulation, PR3 can be released into the extracellular environment [3]. The proteolytic activity of PR3 against a variety of extracellular matrix proteins including elastin, fibronectin, collagen type IV and laminin, points to important physiological or pathological functions of the enzyme [4–6]. This has been illustrated by the observation that PR3 can cause emphysema after intratracheal instillation in hamsters, more profound than human neutrophil elastase (HNE) [1]. PR3 has been cloned and the active site showed resemblance with the active site of HNE [7]. We have recently investigated the extended substrate-binding sites of both enzymes and found that these are significantly different [8].

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PR3 has been identified as the antigen that is recognized by circulating C-ANCA in the serum of patients suffering from Wegener's granulomatosis (WG), a disease characterized by granulomatous inflammation, necrotizing and crescentic glomerulonephritis, and vasculitis [3,9–11]. The hypothesis that C-ANCA might be involved in the pathogenesis of WG is supported by the observations that C-ANCA titre is correlated with WG disease activity, relapses of WG are preceded by recurrence or increase of C-ANCA titre, and treatment based on changes in C-ANCA titre has been reported to be beneficial [12–16].

Different pathophysiologic mechanisms have been proposed [17,18]. Upon inflammatory stimulation of neutrophils, PR3 can be released into the extracellular environment, and attach to the outer membrane of the cells. It has recently been demonstrated that binding of C-ANCA to PR3 on the membrane of activated neutrophils results in production of reactive oxygen species and degranulation [19]. This might cause derailment of the inflammatory process.

Another mechanism might be interference of C-ANCA with PR3 regulation *in vivo*. Recently, we have demonstrated interference of C-ANCA with PR3 proteolytic activity and with PR3 inhibition by its major physiologic inhibitor α_1-antitrypsin (α_1-AT) [20]. Moreover, WG disease activity

appeared to be more closely correlated to C-ANCA inhibitory activity towards PR3–α_1-AT complexation than to the total amount of C-ANCA [21]. However, inhibition of PR3 proteolytic activity by C-ANCA suggests protection against rather than enhancement of tissue damage. The possible pathogenic role of inhibiting C-ANCA might depend on the stability of the PR3–C-ANCA complex. Dissociation of the complex might enable PR3 to cause tissue damage. To elucidate the possible pathogenic role of C-ANCA it is therefore important to know the susceptibility of inhibitory C-ANCA IgG complexed to PR3 for proteolysis by this enzyme. In the present study we have tested the proteolytic activity of PR3 against human IgG and, in particular, against inhibitory C-ANCA IgG upon complexation to the protease.

MATERIALS AND METHODS

Enzymes

PR3 was purified from neutrophil granules by dye-ligand affinity chromatography over Matrix Gel orange A (Amicon Div., Danvers, MA) followed by ion-exchange chromatography over Bio-Rex 70 (BioRad Laboratories, Richmond, CA) as described by Kao *et al.* [1]. Purity of the PR3 preparation was proven by the absence of contaminants in SDS–PAGE analysis. PR3 migrated as a 29-kD triplet [8]. Protein concentration was measured according to Bradford using bovine serum albumin (BSA) as a standard [22]. HNE was purchased from Elastin Products (Pacific, MO). Active site titrations of PR3 and HNE were performed as described [20]. Briefly, active site titrated bovine chymotrypsin was obtained from the National Institute for Biological Standards and Control (UK) and used as a standard to determine the active inhibitor concentration of α_1-AT (Calbiochem, San Diego, CA) by cleavage of MeO-Suc-Arg-Pro-Tyr-pNA (S2586; KabiVitrum, Stockholm, Sweden). This α_1-AT was used as a standard to determine active enzyme concentrations of PR3 and HNE using MeO-Suc-Ala-Ala-Pro-Val-pNA (M4765; Sigma Chemical Co, St Louis, MO) as substrate.

IgG proteins

Human IgG subclass proteins were obtained from the plasma of patients with multiple myeloma. The IgG1, IgG2, IgG3, and IgG4 were isolated by ammonium sulphate precipitation, followed by chromatography on DEAE-Sephadex (Pharmacia LKB, Uppsala, Sweden). C-ANCA IgG was isolated by affinity chromatography as previously described [20]. Briefly, C-ANCA IgG were purified by ammonium sulphate precipitation and subsequent affinity chromatography using protein G Sepharose CL-4B (Pharmacia). C-ANCA-positive sera (C-ANCA IgG) were derived from three patients with active WG. In a previous study these same C-ANCA IgG revealed significant inhibitory activity towards PR3 proteolytic activity [20]. The IgG preparations appeared to be pure by SDS–PAGE analysis. Protein concentrations were measured spectrophotometrically at 280 nm. The C-ANCA IgG reacted specifically with PR3 as determined by antigen capture ELISA, and were radiolabelled with ^{125}I (Radiochemical Centre, Amersham, UK) by the IODO-GEN method [23].

Digestion of IgG

Human IgG subclass proteins (1 mM) were incubated with either PR3 (500 nM) or HNE (500 nM) in PBS for 2·5 h at 37°C. The reaction was stopped by adding diisopropylfluorophosphate (DFP; Aldrich Chemie, Brussels, Belgium) to a final concentration of 1 mM. Samples were diluted in sample buffer that consisted of 4% (w/v) BSA, 1% (w/v) Triton X-100, 0·5% (w/v) SDS, 0·5 M NaCl in PBS, and were subsequently boiled for 5 min at 95°C. Reducing conditions were obtained by adding 1% mercaptoethanol to the sample buffer. The samples were electrophoresed in 10% polyacrylamide gels, and digestion products were visualized by silver staining.

To investigate antigen-complexed C-ANCA proteolysis by PR3, MoAb 12.8 (anti-PR3) IgG1 was purified from ascitic fluid by protein A affinity chromatography, and was coupled covalently to cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) at a concentration of 10 mg/ml (12.8-Sepharose). Radiolabelled C-ANCA IgG were incubated with PR3 in PBS (molar ratio 40 : 1) for 2 h at 4°C. Inactivated PR3, obtained by preincubating PR3 with a 10-fold molar excess of DFP, was used as a control. Subsequently, 50 μl of 12.8-Sepharose were added and complexes were precipitated by rotating end-over-end for 2 h at 4°C. The 12.8-Sepharose was thoroughly washed in 2 M NaCl/PBS, finally resuspended in 1 ml of PBS, and incubated at 37°C. At time intervals $t = 0$, 0·25, 0·5, 1, 2, 4, 8, 24, and 48 h samples (100 μl) of the supernatant were taken and radioactivity was measured in a gamma counter. The supernatants were boiled in sample buffer for 5 min at 95°C, and proteins were analysed by SDS–PAGE (12·5% gels) and radiography (XAR5 film; Eastman Kodak Co, Rochester, NY). At $t = 48$ h the 12.8-Sepharose were boiled for 5 min with sample buffer and centrifuged. The supernatants were analysed as described above.

RESULTS

The reaction mixtures resulting from the incubation of the four human IgG subclasses with PR3 were analysed by SDS–PAGE and stained with silver. The results are shown in Fig. 1. Degradation products of IgG proteolysis by PR3 are indicated by black arrows. In the adjacent lanes, degradation products are shown after incubation of human IgG subclasses with HNE and IgG subclass proteins without the addition of enzymes. All IgG subclasses were cleaved by PR3. Degradation products were different from those obtained with HNE, although bands at similar positions could also be identified. The specificity of PR3-mediated degradation products of each IgG subclass depended on the particular myeloma protein tested. We have observed variations of degradation products between myeloma proteins representing the same IgG subclass. Eight samples were tested, and examples are shown in Fig. 1. The differences in degradation profiles of the same IgG subclass appeared in the low mol. wt (30 000–10 000 D). No consistent pattern of proteolytic fragmentation was observed for any of the subclasses. Human IgG proteolysis by PR3 appeared to be dose-dependent and was completely inhibited by preincubation of PR3 with DFP, suggesting that the proteolytic activity of PR3 is responsible for degrading human IgG (results not shown).

Proteolysis of three radiolabelled C-ANCA IgG complexed to PR3 was analysed by SDS–PAGE and subsequent autoradiography. The results obtained with one C-ANCA IgG are

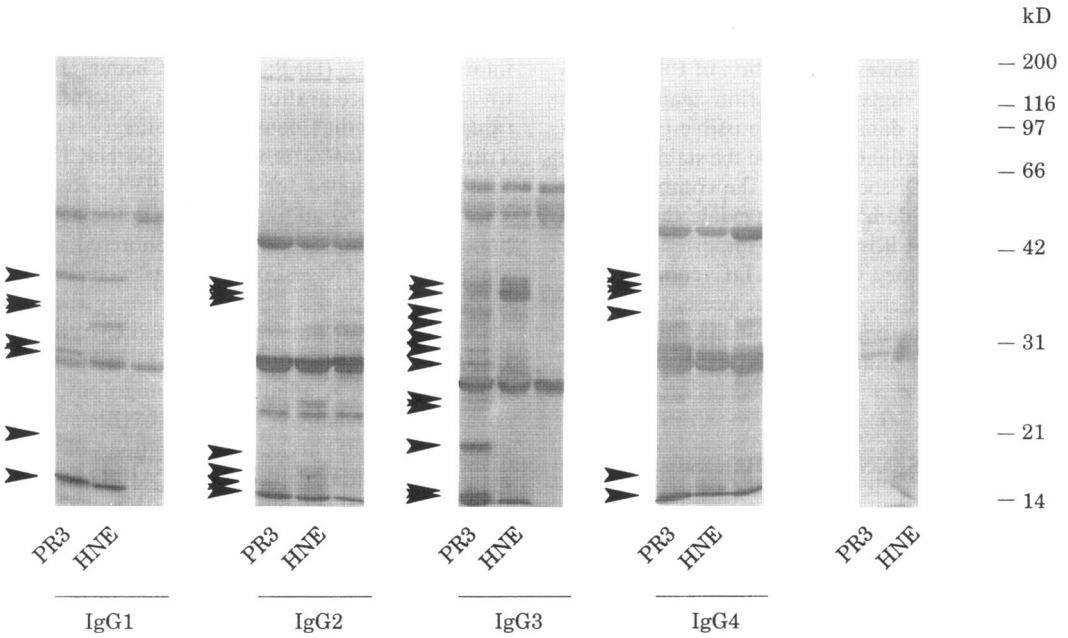

Fig. 1. Analysis of human IgG proteolysis by proteinase 3 (PR3). Human IgG subclass proteins were incubated with either PR3, human neutrophil elastase (HNE) or without enzyme as indicated. After 2·5 h of incubation at 37°C, reaction mixtures were electrophoresed on a SDS–polyacrylamide (10%) gel under reducing conditions. Subsequently proteins in the gel were stained with silver. Degradation products as a result of IgG subclass cleavage by PR3 are indicated by black arrows.

shown in Fig. 2 and are representative for the results obtained with C-ANCA IgG from the other two WG patients. After incubation of radiolabelled C-ANCA IgG with active PR3, complexes were immunoprecipitated and subsequently incubated at 37°C. At time intervals as indicated in Fig. 2, supernatants were analysed by SDS–PAGE followed by autoradiography. In Fig. 2a supernatant was analysed after immunoprecipitation of C-ANCA IgG complexed to active PR3. Figure 2b shows analysis of the supernatant when C-ANCA IgG was complexed to DFP-inactivated PR3. Purified C-ANCA IgG under identical conditions but not complexed to (i.e. in the absence of) PR3 is shown in Fig. 2c.

In Fig. 2a at $t = 0$ h, no radioactivity could be detected in the supernatant. At $t = 0{\cdot}25$ h, small peptides were detected that migrated at the bottom of the gel. Intermediate products were not detected (not shown). Between $t = 0{\cdot}25$ h and $t = 48$ h the amount of degraded material increased in time. This is also illustrated by the amount of radioactivity measured in the supernatant, as shown in Fig. 2d. The peptides migrated in the gel as two different bands, one with a mol. wt of approximately 14 000 D, and a second band with a lower mol. wt representing very small peptides present in the dye-front of the gel. In Fig. 2b, no degradation products were detected and no increase of radioactivity was measured in the supernatant (Fig. 2e), suggesting that proteolysis of complexed C-ANCA IgG was due to proteolytic activity of PR3. Degradation products of C-ANCA IgG as a consequence of the conditions used in these experiments could be excluded by incubation in the absence of PR3 (Fig. 2c).

In Figs 2a,b small amounts of intact C-ANCA IgG were noticed, probably due to low-affinity binding of some of the IgG and its release into the supernatant under the experimental conditions.

After $t = 48$ h complexed C-ANCA IgG were boiled from the Sepharose beads and analysed as described above. The results are shown in Fig. 2f. C-ANCA IgG complexed to inactivated PR3 (Fig. 2f, lane 2) appeared to be identical to the original C-ANCA IgG (Fig. 2f, lane 1). However, C-ANCA IgG complexed to active PR3 migrated as a diffuse band of approximately 150 000 D, together with small peptides at the bottom of the gel (Fig. 2f, lane 3), suggesting that the degradation products detected in the supernatant indeed originated from complexed C-ANCA IgG.

DISCUSSION

In the present study we demonstrate that neutrophil PR3 exhibits proteolytic activity towards all human IgG subclass proteins. The molar ratios used may be easily achieved under *in vivo* conditions of inflammation and neutrophil activation [24]. Since human IgG has important physiologic functions, such as opsonic activity, specificity for mononuclear cells, and binding to complement, proteolysis by neutrophil PR3 can be important under both physiological and pathophysiological conditions. The *in vivo* prospects of the C-ANCA IgG–PR3 complexes might either be limited by proteolysis and dissociation, opsonization with complement, phagocytosis by neutrophils, or destruction by macrophages of the reticuloendothelial system in the liver and spleen.

The degradation profiles of human IgG subclasses and C-ANCA IgG complexed to PR3 differed. Degradation of C-ANCA IgG complexed to PR3 revealed no large intermediate products. This might be explained, at least in part, by the different detection methods. Breakdown products of subclass IgG were visualized by silver staining. ^{125}I C-ANCA IgG was used to visualize breakdown of C-ANCA IgG complexed to PR3 for better quantification and because of higher sensitivity. In this technique, however, only ^{125}I-labelled

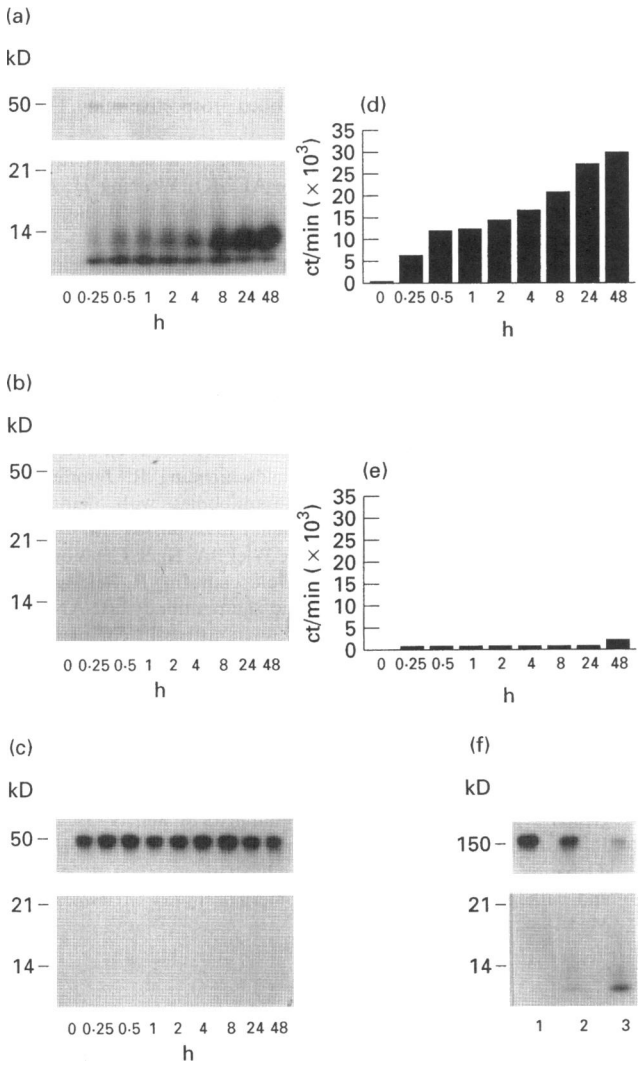

Fig. 2. Analysis of C-ANCA IgG proteolysis by proteinase 3 (PR3) when complexed to the enzyme. C-ANCA IgG was radiolabelled and incubated with either active PR3, diisopropylfluorophosphate (DFP)-inactivated PR3, or without enzyme. Complexes were immunoprecipitated and subsequently incubated at 37°C. At time intervals as indicated a sample (100 μl) of the supernatant was analysed by SDS–PAGE followed by autoradiography. (a) Analysis of the supernatant after immunoprecipitation of C-ANCA IgG complexed to active PR3. (b) Analysis of the supernatant after immunoprecipitation of C-ANCA complexed to DFP-inactivated PR3. (c) Analysis of C-ANCA IgG under identical conditions but not complexed to PR3. (d, e) Radioactivity measured in the supernatant after immunoprecipitation of C-ANCA complexed to active PR3 and DFP-inactivated PR3, respectively, at time intervals as indicated. (f) SDS–PAGE analysis of the immunoprecipitated C-ANCA IgG after 48 h of incubation. Uncomplexed C-ANCA IgG (lane 1) was compared with C-ANCA IgG complexed to DFP-inactivated PR3 (lane 2) and C-ANCA IgG complexed to active PR3 (lane 3). In a, b, c, and f no intermediate products were detected (not shown).

fragments can be detected, and only if dissociated from the complex, leaving larger fragments, still attached to the Sepharose-bound PR3, unnoticed.

In particular the finding that also human IgG with specificity for PR3 (C-ANCA) is cleaved when complexed to the enzyme may have implications for the possible pathogenic role of C-ANCA in WG.

In a previous study we have shown that binding of C-ANCA to PR3 prevents the enzyme from being inactivated by its major physiologic inhibitor, α_1-AT [20]. This interference of PR3–α_1-AT complexation by C-ANCA was not only observed with purified IgG, but also after addition of PR3 to C-ANCA-positive sera, suggesting that also *in vivo* C-ANCA inhibit PR3–α_1-AT complexation. Furthermore, the inhibitory effect of C-ANCA on PR3–α_1-AT complexation correlated with WG disease activity [21]. These findings suggest that C-ANCA may be involved in the pathogenesis of WG by interference with PR3–α_1-AT complexation.

Another pathophysiologic mechanism might be C-ANCA interference with PR3 activity, as we have recently described [20]. C-ANCA inhibition of PR3 proteolytic activity suggests protection against, rather than enhancement of tissue injury. However, in the present study we demonstrate that PR3 inhibition by C-ANCA is only a transient effect. C-ANCA complexed to PR3 remains a target of proteolytic attack by PR3. This might lead to further dissociation of PR3–C-ANCA complexes, and to the subsequent release of active PR3 that could cause tissue damage, possible at sites of immune complex depositions that might be remote from the initial sites of inflammation where these complexes are formed.

Finally, proteolysis of C-ANCA IgG complexed to PR3 might help to explain why only minimal amounts of immune deposits can be detected in renal lesions of ANCA-related glomerulonephritis. This pauci-immunity has been ascribed to rapid clearance of the immune complexes from the glomerular basement membrane [17]. Thus, C-ANCA IgG proteolysis by PR3 might, in a number of different ways, have considerable implications for the possible role of these autoantibodies in the pathogenesis of WG.

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