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Bioactive Proteinase 3 on the Cell Surface of Human Neutrophils: Quantification, Catalytic Activity, and Susceptibility to Inhibition[1]

Edward J. Campbell, Melody A. Campbell, and Caroline A. Owen[2]

Although proteinase 3 (PR3) is known to have the potential to promote inflammation and injure tissues, the biologic forms and function of PR3 in polymorphonuclear neutrophils (PMN) from healthy donors have received little attention. In this paper, we show that PMN contain 3.24 ± SD 0.24 pg of PR3 per cell, and that the mean concentration of PR3 in azurophil granules of PMN is 13.4 mM. Low levels of PR3 are detectable on the cell surface of unstimulated PMN. Exposure of PMN to cytokines or chemoattractants alone induces modest (1.5- to 2.5-fold) increases in cell surface-bound PR3. In contrast, brief priming of PMN with cytokines, followed by activation with a chemoattractant, induces rapid and persistent, 5- to 6-fold increases in cell surface expression of PR3, while causing minimal free release of PR3. Membrane-bound PR3 on PMN is catalytically active against Boc-Alanine-Alanine-Norvaline-thiobenzyl ester and fibronectin, but in marked contrast to soluble PR3, membrane-bound PR3 is resistant to inhibition by physiologic proteinase inhibitors. PR3 appears to bind to the cell surface of PMN via a charge-dependent mechanism because exposure of fixed, activated PMN to solutions having increasing ionic strength results in elution of PR3, HLE, and CG, and there is a direct relationship between their order of elution and their isoelectric points. These data indicate that rapidly inducible PR3 expressed on the cell surface of PMN is an important bioactive form of the proteinase. If PR3 expression on the cell surface of PMN is dysregulated, it is well equipped to amplify tissue injury directly, and also indirectly via the generation of autoantibodies. ***The Journal of Immunology,*** **2000, 165: 3366–3374.**

Proteinase 3 (PR3, EC 3.4.21.37)[3] is a cationic serine proteinase that is contained within the azurophil granules of human polymorphonuclear neutrophils (PMN) along with human leukocyte elastase (HLE) and cathepsin G (CG) (1). Although the azurophil granules are the major intracellular store of PR3 in PMN, PR3 has also recently been reported to be localized within the secretory and specific granules of PMN (2). PR3 is also expressed by monocytes, basophils, and mast cells (3). To date, most of the interest in this proteinase has focused on its role in the pathogenesis of Wegener's granulomatosis. Most patients with active Wegener's granulomatosis have circulating anti-neutrophil cytoplasmic Abs (ANCA), and PR3 is the most common antigenic target for ANCA in this disease (4). Circulating PMN in patients with active Wegener's granulomatosis express PR3 on their cell surface, and the binding of PR3-ANCA to membrane-bound PR3 is followed by ligation of PMN Fcγ receptors by the Fc component of ANCA, which is a potent stimulus for the respiratory burst and PMN degranulation (5). The reactive oxygen species and proteinases that are released from PMN under these conditions may contribute to the aggressive vascular inflammation and injury that is characteristic of Wegener's granulomatosis.

Despite the intense interest in membrane-bound PR3 on PMN in Wegener's granulomatosis, remarkably little is known about the localization, activities, and roles of PR3 in inflammatory cells from healthy subjects. Studies of purified PR3 have shown that the soluble form of the proteinase can degrade a number of extracellular matrix macromolecules and heat shock proteins (6), and that it can also cleave and activate the precursor forms of cytokines (7, 8), including IL-8, IL-1β, and TNF-α. However, little is known about the mechanisms by which PR3 might first come into contact with the immune system in normal individuals. Although PR3 has been identified on the surface of human PMN (9–11), there have been no studies of the quantity of enzyme or its catalytic activity. In this respect, it is noteworthy that previous work from our laboratory has shown that activation of PMN with cytokines and chemoattractants results in striking up-regulation of catalytically active HLE and CG on the cell surface of PMN from healthy donors, and that membrane-bound HLE and CG are both remarkably resistant to inhibition by physiologic inhibitors (12–15).

In this paper, we have studied bioactive forms of PR3 in PMN from healthy individuals. Our results have shown that PMN contain PR3 at mM concentrations within their azurophil granules, and that PR3 is also expressed on the cell surface of PMN. Expression of PR3 at the cell surface of PMN is rapidly and strikingly up-regulated by proinflammatory mediators, and this form of PR3 is catalytically active yet substantially resistant to inhibition by naturally occurring proteinase inhibitors. These data indicate that, when localized to the cell surface of PMN, PR3 may have important biologic activities during physiologic processes, as well as in the pathogenesis of diseases such as Wegener's granulomatosis.

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[3] Abbreviations used in this paper: PR3, proteinase 3; α_1-PI, α_1-proteinase inhibitor; ANCA, anti-neutrophil cytoplasmic Abs; CG, cathepsin G; HLE, human leukocyte elastase; PAF, platelet activating factor; PMN, polymorphonuclear neutrophils; SLPI, secretory leukocyte proteinase inhibitor; Boc-Ala-Ala-Nva-SBzl, Boc-Alanine-Alanine-Norvaline-thiobenzyl ester; CG/CMK, Z-Gly-Leu-Phe-choromethyl ketone.

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Materials and Methods

Materials

Human serum albumin was obtained from the American Red Cross (Washington, DC). 1-*O*-Hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine (platelet activating factor (PAF)) was purchased from Bachem (Torrance, CA). TNF-α was obtained from Genzyme (Boston, MA). HBSS was purchased from Life Technologies (Grand Island, NY). Permount was purchased from Fisher Scientific (Pittsburgh, PA). Goat anti-rabbit IgG conjugated to rhodamine and goat anti-rabbit conjugated to HRP were obtained from Cappel (Durham, NC). Goat anti-rabbit IgG conjugated to colloidal gold particles was purchased from Chemicon International (Temecula, CA). Purified human PR3, rabbit antiserum to human PR3, and Boc-Alanine-Alanine-Norvaline-thiobenzyl ester (Boc-Ala-Ala-Nva-SBzl) were purchased from Elastin Products (Owensville, MO). Rabbit antiserum to HLE and control (preimmune) rabbit serum were obtained from Zymed (San Francisco, CA). Polyclonal rabbit anti-human CG was purchased from Dako (Carpinteria, CA).

Z-Gly-Leu-Phe-choromethyl ketone (CG/CMK), methoxy-succinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin, and succinyl-Ala-Ala-Phe-7-amino-4-trifluoromethyl coumarin were purchased from Enzyme Systems Products (Livermore, CA). α_1-Proteinase inhibitor (α_1-PI) was obtained from Bayer (New Haven, CT). Secretory leukocyte proteinase inhibitor (SLPI) was obtained from Amgen (Boulder, CO). Recombinant human elafin was a generous gift from Dr. J. M. Sallenave (Rayne Laboratory, Department of Medicine, Edinburgh University, Scotland, U.K.). All other reagents were purchased from Sigma (St. Louis, MO).

PMN isolation

Human PMN (>95% pure) were obtained from peripheral blood of healthy donors using the Ficoll-Hypaque technique (16). Extracts of PMN were prepared in 0.05 M phosphate buffer (pH 7.4) containing 0.04% (v/v) Triton and 1 M NaCl.

Competitive binding ELISA for PR3, HLE, and CG

Microtiter plates (Nunc, Naperville, IL) were coated with 1 μg of PR3 in 10 mM phosphate buffer containing 0.6 M NaCl (pH 7.4) overnight at 4°C, washed three times to remove unbound protein, then incubated for 1 h at 37°C with 100 ml 1% (w/v) BSA in 10 mM phosphate buffer containing 0.6 M NaCl, and 0.05% (v/v) Tween to block additional protein binding sites. PR3 standards and unknowns were incubated overnight at 4°C in a volume of 150 μl in polypropylene radioimmunoassay vials (Starstedt, Princeton, NJ) with 50 μl of rabbit antiserum to PR3 (diluted 1:1600). A total of 100 μl of each sample were transferred to the Ag-coated wells and incubated for 90 min at 4°C. The plates were washed three times and incubated for 60 min at 37°C with 100 ml HRP-conjugated goat anti-rabbit IgG diluted 1:4000. The assay was developed using *o*-phenylenediamine in 50 mM citrate (pH 4.5), and the reaction was stopped using 25 μl 2 N H_2SO_4. Absorbances were read using an automated microtiter plate reader (Molecular Devices, Palo Alto, CA). The assay was sensitive to 4 ng PR3/ml. Competitive binding ELISAs for HLE and CG were performed, as described previously (17).

PMN stimulation and fixation

PMN were resuspended at 5×10^6/ml in HBSS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}, then incubated at 37°C for 15 min with or without PMA (50 ng/ml) or A23187 (1 μM). Cells were also incubated at 37°C for 5 min with 5 μg/ml cytochalasin B then for 10 min with 10^{-8} M fMLP. We also assessed the effects of biologically relevant mediators. Cells were incubated for 30 min with or without 10^{-9} M PAF, 100 U/ml TNF-α , 100 ng/ml bacterial LPS from *Escherichia coli* 0111:B4, 10^{-8} M IL-8, or 10^{-8} M fMLP. Cells were also primed for 15 min with 10^{-9} M PAF, 100 U/ml TNF-α, 100 ng/ml LPS, then activated for 30 min with 10^{-8} M fMLP or 10^{-8} M IL-8. Following activation, cells were fixed for 3 min at 4°C with PBS containing 3% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde, and then washed in HBSS. We have shown previously that these concentrations of cytokines and chemoattractants do not adversely affect PMN viability (12, 14).

Immunofluorescence staining

We used quantitative immunofluorescence microscopy to assess the effects of agonists on cell surface expression of PR3. Our previous work has confirmed that this technique detects serine proteinases on the cell surface of PMN, rather than intracellular enzymes (13). Fixed cells were incubated at 4°C in HBSS containing 1% (v/v) human serum albumin and 50 μg/ml goat IgG to reduce nonspecific binding of Abs. To stain for membrane-bound PR3, the PMN were incubated for 45 min at 4°C with rabbit antiserum to PR3 or control (preimmune) rabbit serum, then washed twice to remove excess Ab. Cells were then incubated for 45 min at 4°C with rhodamine-conjugated goat anti-rabbit IgG and washed twice in HBSS. Cytocentrifuge preparations were mounted in 25% (v/v) glycerol in PBS containing 250 μg/ml *p*-phenylenediamine to reduce photobleaching, and coverslips were applied.

The immunostained cells were examined with phase-contrast and incident-light fluorescence microscopy (Leitz Dialux 20 with L2 filter set and Leitz NPL Fluotar 40×, N.A. 1.32 objective; Leica, Deerfield, IL). Cell surface immunofluorescence was quantified by image analysis using MetaMorph software, (Universal Imaging, Westchester, PA) as described previously (13). The fluorescence intensity of 100–150 cells in each group was quantified. To correct for nonspecific staining, the mean fluorescence value for the group of cells that were incubated with the control rabbit serum was subtracted from each of the values for cells that were incubated with the rabbit antiserum to PR3, and the mean specific fluorescence value was determined. To determine the percentage of cells that express PR3, we calculated the percentage of cells that were stained with the antiserum to PR3, that had an integrated fluorescence value greater than the mean fluorescence + 3 SD of the cells that were incubated with the control rabbit serum.

Immunogold staining of PMN

Unstimulated cells, PMN activated with A23187, or PMN primed with LPS then activated with IL-8 were fixed then incubated with rabbit antiserum to PR3 or control rabbit serum, as described above. The cells were then incubated with goat anti-rabbit IgG conjugated to colloidal gold particles (diameter 20 nm) and washed twice in HBSS. Cytocentrifuge preparations were mounted in Permount, coverslips were applied, and the cells were examined by phase-contrast and polarization reflection microscopy (polarized incident light, when reflected from gold particles, passes through the cross-polarizer in the Leitz RK reflection contrast 100×, N.A. 1.32 objective).

Quantification of free release of PR3 by activated PMN

PR3, HLE, and CG were quantified in cell-free supernatant fluids from primed and stimulated PMN and in cell extracts of unstimulated PMN (prepared in HBSS containing 1 M NaCl and 0.04% (v/v)Triton) using competitive binding ELISAs, as described above.

Catalytic activity of membrane-bound PR3

To inactivate proteinases expressed endogenously on the surface of PMN, unstimulated PMN were fixed then heated to 100°C for 5 min and chilled on ice. The cells were incubated overnight at 4°C with 1 mM PMSF, then washed three times in HBSS to remove PMSF. We confirmed that the cells were intact when examined by phase contrast microscopy, and had no activity against Boc-Ala-Ala-Nva-SBzl, a synthetic substrate for PR3 (as described below), methoxy-succinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin, or succinyl-Ala-Ala-Phe-7-amino-4-trifluoromethyl coumarin, synthetic fluorogenic substrates which are specific for HLE and CG, respectively, as described previously (14, 15).

The cells were then incubated overnight at 4°C with purified PR3 (1 μg/10^6 cells), fixed, then washed three times to remove unbound enzyme. We have shown previously that our fixation process does not affect the catalytic activity of membrane-bound serine proteinases (13, 15). Membrane-bound PR3 activity was assayed using Boc-Ala-Ala-Nva-SBzl as the substrate (18). Briefly, 10^6 PMN or 3.1–80 ng soluble PR3 in a volume of 100 ml HBSS were added to 900 ml of 0.5 M NaCl containing 0.1 M HEPES and 400 μM 4′,4′-dithiodipyridine and 100 μM Boc-Ala-Ala-Nva-SBzl (pH 7.5). The samples were incubated for 2 h at 37°C, then the absorbances of cell-free supernatant fluids were determined at 324 nM using a DU8 spectrophotometer (Beckman Instruments, Palo Alto, CA).

To assess whether membrane-bound PR3 is active against soluble human fibronectin, exogenous PR3 bound to fixed PMN, fixed PMN incubated without PR3 (both at 2.5×10^6/assay), or soluble PR3 (125 ng/assay) were incubated overnight in 50 μl HBSS with 25 μg soluble human fibronectin. To confirm that there was no detachment of PR3 from the surface of PMN, we incubated PMN that bound exogenous PR3 overnight at 37°C in HBSS alone, then harvested the cell-free supernatant fluid, and incubated the latter with soluble human fibronectin, as described above. Cell-free supernatant fluids were reduced with 10% (v/v) β-mercaptoethanol, then were subjected to 7.5–15% gradient SDS-PAGE (13). To provide further assurance that the fibronectin-degrading activity associated with the cells was due to membrane-bound PR3, we incubated soluble PR3 (125 ng/assay) or exogenous PR3 that was bound and fixed onto the cell surface of PMN both in the presence and absence of 2 μM elafin or SLPI in a total

volume of 50 μl HBSS for 15 min at 25°C. The samples were then incubated with soluble human fibronectin, and cell-free supernatant fluids were analyzed by SDS-PAGE, as described above.

Susceptibility of membrane-bound PR3 to inhibition

Purified PR3 (25 ng) or exogenous PR3, which was bound and fixed onto the cell surface of PMN (10^6/assay), were incubated at 25°C for 15 min in an assay volume of 100 ml HBSS with or without the following inhibitors: 1) 5 μM α_1-PI, 2) 2 μM SLPI, 3) 2 μM elafin, 4) 1 mM PMSF, 5) 100 μM CG/CMK, or 6) 2 μM α_1-antichymotrypsin. Residual PR3 activity was quantified in cell-free supernatant samples using Boc-Ala-Ala-Nva-SBzl, as described above. The results were expressed as percent inhibition.

Elution of PR3, HLE, and CG from the cell surface of activated PMN with increasing ionic strength

PMN were optimally primed with PAF and stimulated with fMLP, then fixed (to prevent leakage of intracellular proteinases; Refs. 12 and 14), as described above. Cells were resuspended at 6×10^6/ml in PBS containing 0.15, 0.3, 0.5, 0.75, 1.0, and 1.25 M NaCl. The cells were incubated overnight at 4°C on an orbital rotator. Cells were also resuspended in PBS containing 0.15 M NaCl, and incubated at 37°C for up to 60 min. Cell-free supernatant fluids and cell extracts of unstimulated PMN were assayed for immunoreactive PR3, HLE, and CG using competitive binding ELISAs, as described above.

Statistics

Data are expressed as mean ± SEM or mean ± SD. The results for paired and unpaired data were compared using the Student *t* test for parametric data and the Mann-Whitney rank sum test for nonparametric data; *p* values <0.05 were considered significant.

Results

Quantification of PR3 in human PMN

We found that PMN contain 3.24 ± SD 0.24 μg of PR3 per 10^6 cells (or 3.24 pg per cell; $n = 17$ donors). Assuming that HLE and PR3 reside in the same azurophil granules, we can use our data together with the mean number of HLE-containing azurophil granules per PMN (399 ± (SEM) 20; Ref. 19), and the mean volume of a single azurophil granule (2.09×10^{-17} L; Ref. 20), to calculate that, on average, each azurophil granule contains 8.12 fg (168,644 molecules) of PR3 at a mean concentration of 13.4 mM.

Cell surface expression of PR3 in response to pharmacologic agonists

We first tested the effects of pharmacologic agonists that are potent inducers of PMN degranulation (PMA, cytochalasin B and fMLP, and the calcium ionophore, A23187). Unstimulated cells had low, but detectable levels of PR3 on their cell surface (Fig. 1*A*). Activation of cells with all of the pharmacologic agents tested induced highly significant increases in cell surface expression of PR3. A23187 was consistently the most potent inducer of the expression of PR3 on the surface of PMN, inducing ~10-fold increases in expression of PR3 when compared with unstimulated cells.

Cell surface expression of PR3 in response to proinflammatory mediators

Activation of cells with optimal concentrations of PAF, TNF-α, LPS, or IL-8 alone induced modest 1.5- to 2.5-fold increases in cell surface expression of PR3 on PMN (Fig. 1*B*). However, synergistic increases in cell surface expression of PR3 were observed when cells were first primed for 5–15 min with LPS, PAF, or TNF-α, then activated for 30 min with IL-8 (conditions that induce optimal expression of HLE and CG on the surface of PMN (12, 14)). Primed and activated PMN expressed 5- to 6-fold more PR3 on their cell surface when compared with unstimulated cells. Similar results were obtained when cells were primed with cytokines, then activated with fMLP (data not shown). The priming effects of cytokines were rapid and persistent. Following priming of cells with

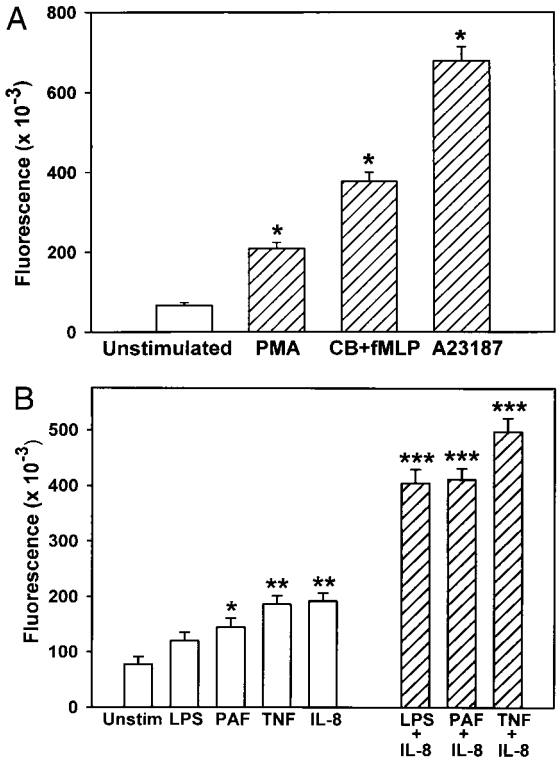

FIGURE 1. *A*, Effect of pharmacologic agonists on cell surface expression of PR3 by PMN. PMN were incubated for 15 min with or without 50 ng/ml PMA, 1 μM A23187, or for 5 min with 5 μg/ml cytochalasin B then for 10 min with 10^{-8} M fMLP (*CB + fMLP*). Cells were then fixed and stained with rhodamine for cell surface PR3. Fluorescence was quantified by analysis of images captured by a chilled charge-coupled device imager, as described in *Materials and Methods*. Note that all of the agonists induced highly significant increases in cell surface expression of PR3, when compared with unstimulated cells. Data are mean ± SEM; $n = 100$–150 cells. *, $p < 0.001$ when compared with unstimulated cells. *B*, Effect of biologically relevant signals on cell surface expression of PR3 on PMN. PMN (□) were incubated for 30 min with or without optimal concentrations of TNF-α, LPS, PAF, or IL-8. Cells (▨) were also primed for 5–15 min with the same concentrations of LPS, TNF-α, or PAF, then stimulated for 30 min with 10^{-8} M IL-8. PMN were then fixed and immunostained with rhodamine for cell surface-bound PR3. Note that the agonists when used alone induced modest (1.5- to 2.5-fold) increases in cell surface expression of PR3 when compared with unstimulated cells, but priming of cells with cytokines followed by activation with IL-8 resulted in synergistic increases in cell surface expression of PR3. Data are mean values; error bars represent SEM; $n = 100$–150 cells. *, $p = 0.007$; **, $p < 0.001$ compared with unstimulated cells; and ***, $p < 0.001$ compared with cells incubated with IL-8 alone.

TNF-α, significant increases in cell surface expression of PR3 were observed within 1 min, and persisted for at least 90 min after exposure to IL-8 (data not shown).

In addition to increasing the mean quantity of PR3 on the cell surface of PMN, priming of cells with cytokines followed by activation of cells with IL-8 also increased the percentage of cells that express PR3. We found that 38.0 ± (SEM) 13.7% (9.5% to 85.0%) of unstimulated cells expressed PR3 compared with 65.1 ± 9.1% ($p = 0.062$), 70.7 ± 11.4% ($p = 0.014$), and 78.1 ± 6.9% ($p = 0.008$) of cells primed with LPS, PAF, and TNF-α, respectively, then activated with IL-8 ($n = 5$ donors). Together, these data indicate that exposure of cells to proinflammatory mediators induces rapid and persistent increases in the mean quantity of PR3 on the cell surface of PMN as well as significant increases in the percentage of cells that express PR3 on their cell surface.

Immunogold localization of PR3 on the cell surface of activated PMN

To exclude the possibility that our immunofluorescence technique was detecting intracellular PR3, we localized PR3 immunoreactivity using a second Ab conjugated to colloidal gold particles with a diameter of 20 nm (which are too large to penetrate cells). There were a few gold particles associated with unstimulated cells that were stained for cell surface-bound PR3 (Fig. 2). In marked contrast, cells that were activated with either A23187 or LPS and IL-8, then stained for PR3 had numerous gold particles localized to their cell surface. There was almost a complete lack of gold particles associated with LPS and IL-8 activated cells (Fig. 2) or A23187-activated cells (not shown) that were incubated with the control rabbit serum. These data confirm that PR3 translocates to the external surface of the plasma membrane of PMN following cellular activation.

Free release of PR3 during cellular activation

To quantify free release of PR3 in response to cellular activation, PMN were optimally primed with cytokines and activated with IL-8. Immunoreactive PR3, HLE, and CG were then quantified in cell-free supernatant fluids and also in cell extracts of unstimulated PMN using competitive binding ELISAs. Only ~2–3% of the cellular content of PR3 in unstimulated cells was released from PMN during activation, and this was similar to the quantities of HLE and CG that were freely released from cells under the same conditions (Table 1).

Binding of exogenous PR3 to the surface of PMN

To provide further assurance that PR3 can bind to the external surface of the plasma membrane of PMN, we tested whether exogenous PR3 can bind to viable PMN. Cells were incubated at 4°C with or without varying concentrations of soluble PR3, then cell surface-bound PR3 was quantified using immunofluorescence staining and image analysis. Cells that were exposed to exogenous PR3 expressed significantly more cell surface-bound PR3 when compared with cells incubated without PR3, and exogenous PR3 bound to viable PMN in a dose-dependent manner (Fig. 3). These data confirm that PR3 can bind to sites on the external surface of the plasma membrane of PMN.

Catalytic activity of membrane-bound PR3

We assessed whether PR3 on the surface of PMN is catalytically active against Boc-Ala-Ala-Nva-SBzl, a synthetic substrate that is cleaved by PR3. For these studies, it was not possible to study PR3 that is endogenously expressed on cells following activation because HLE and CG are also expressed on the surface of activated PMN (12–14), and there is no substrate available that is specific for PR3 activity. To circumvent this problem, we studied the catalytic activity of exogenous PR3 bound and fixed onto unstimulated cells, as described in *Materials and Methods*.

Fixed PMN that were not exposed to PR3 had no detectable activity against Boc-Ala-Ala-Nva-SBzl (a chromogenic substrate which is cleaved by PR3), or against methoxy-succinyl-Ala-Ala-Pro-Val-7-amino-4-trifluoromethyl coumarin or succinyl-Ala-Ala-Phe-7-amino-4-trifluoromethyl coumarin (fluorogenic substrates that are specific for HLE and CG, respectively). Cells that bound exogenous PR3 had activity equivalent to 45.5 ± (SD) 13.9 ng of soluble PR3 per 10^6 cells ($n = 8$ experiments). These data indicate that cell surface-bound PR3 on PMN is catalytically active against an oligopeptide substrate.

Next, we tested whether cell surface-bound PR3 on PMN can degrade human fibronectin, a component of the extracellular matrix and basement membranes. We incubated the following with soluble human fibronectin for 3 h: 1) exogenous PR3 bound and fixed onto PMN; 2) fixed PMN that were incubated without exogenous PR3 (as a control); or 3) soluble PR3. Cell-free supernatant fluids were then reduced and subjected to SDS-PAGE. Soluble PR3 and exogenous PR3 bound to PMN completely or substantially degraded the fibronectin substrate (Fig. 4, *A* and *B*). In marked contrast, there was no degradation of fibronectin that was incubated with the control PMN that were not exposed to PR3 (Fig. 4*A*). When cell-free supernatant fluids were harvested from the PMN that bound exogenous PR3, then incubated with fibronectin under the same conditions as the cells, no degradation of the fibronectin was observed (Fig. 4*A*). Together, these data indicate that fixed PMN that bind exogenous PR3 to their cell surface can degrade fibronectin, and that this activity is mediated by cell surface-bound PR3, and is not due to leakage of intracellular proteinases, or detachment of PR3 from the surface of the fixed PMN.

To provide further assurance that the fibronectin-degrading activity, which is associated with PMN that bind exogenous PR3, is due to cell surface-bound PR3, we incubated PMN that bound exogenous PR3, or soluble PR3 with fibronectin both in the absence and presence of elafin (which is an effective inhibitor of HLE, CG and PR3), or SLPI (which inhibits HLE and CG, but not PR3 (21, 22)). As expected, elafin inhibited fibronectin degradation by soluble PR3, and by exogenous membrane-bound PR3 on PMN (Fig. 4*B*). In contrast, SLPI was ineffective against either soluble or membrane-bound PR3. Together, these data confirm that membrane-bound PR3 on PMN degrades fibronectin.

Membrane-bound PR3 is resistant to inhibition by high molecular mass inhibitors

We compared the susceptibility of soluble vs membrane-bound PR3 to inhibition by proteinase inhibitors that vary in their molecular size using Boc-Ala-Ala-Nva-SBzl as the substrate. All of the inhibitors of PR3 that were tested (PMSF, elafin, and α_1-PI) were effective inhibitors of soluble PR3 (Fig. 5). As expected, SLPI did not inhibit soluble PR3. PMSF, a low molecular mass inhibitor of serine proteinases (M_r = 174 Da), was almost fully effective against membrane-bound PR3. In contrast, the naturally occurring inhibitors elafin (M_r = 6 kDa) and α_1-PI (M_r = 52 kDa) were only partially effective against membrane-bound PR3 despite the fact that these inhibitors were used in the assay at a 113 ± SD 25.4-fold and a 282.9 ± 63.5-fold molar excess over membrane-bound PR3, respectively. As controls, we also tested CG/CMK and α_1-antichymotrypsin (which are low and high molecular mass inhibitors of CG, respectively). As expected, the inhibitors of CG were ineffective against both soluble and membrane-bound PR3. These data indicate that membrane-bound PR3 on PMN is substantially resistant to inhibition by physiologic inhibitors of PR3.

PR3 binds to the cell surface of PMN following degranulation by a charge-dependent mechanism

We assessed the ability of solutions with increasing ionic strength to elute PR3 from the cell surface of PMN that had been optimally activated to induce cell surface expression of PR3, then fixed to prevent release of intracellular proteinases (12, 14). We compared elution of PR3 to that of HLE and CG, which are both more highly positively charged than PR3 and are known to bind to the PMN cell membrane via ionic interactions (12, 14, 23). Increasing the ionic strength of the solution to 0.75 M NaCl resulted in maximal elution of PR3 from the cell surface of PMN (Fig. 6*A*). As expected, HLE required higher concentrations of NaCl (up to 1 M) for maximal elution, and elution of CG (which is the most cationic of the three proteinases) was not complete even when cells were exposed to 1.25 M NaCl. These results did not represent leakage of

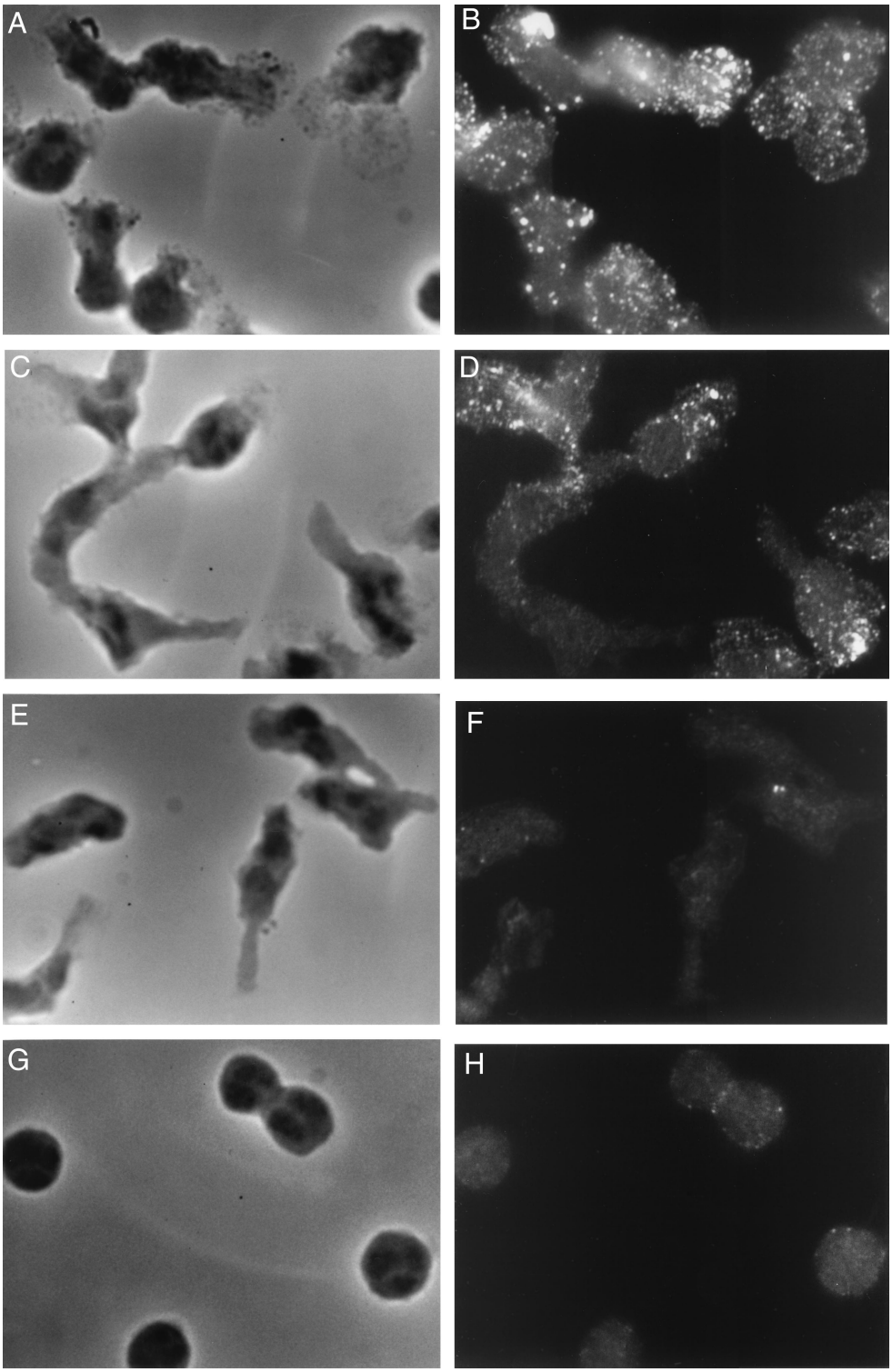

FIGURE 2. Immunogold staining of PMN for cell surface-bound PR3. PMN were activated with A23187 (*A* and *B*), or primed with LPS and activated with IL-8 (*C–F*), or incubated in the absence of agonists (*G* and *H*), as described in *Materials and Methods*. Cells were fixed then incubated with rabbit antiserum to PR3 (*B*, *D*, and *H*), or with nonimmune rabbit serum as a control (*F*), then with goat-anti-rabbit IgG conjugated to colloidal gold particles. *Left*, Cells examined by phase-contrast microscopy. *Right*, The same microscopic fields examined by reflection polarization microscopy. Note that there are a few gold particles associated with unstimulated cells (*H*) but there are numerous gold particles associated with activated PMN (*B* and *D*) that were stained for cell surface PR3. Note the almost complete lack of gold particles associated with LPS and IL-8-activated cells incubated with the control rabbit serum (*F*).

intracellular enzyme into supernatant fluids, because less than 0.4% of the PMN cellular content of lactate dehydrogenase activity is detected in cell-free supernatant fluids when cells are incubated under these conditions (12, 14).

Also noteworthy in Fig. 6*A* is that exposure of PMN to 0.15 M NaCl resulted in significantly greater elution of PR3 from the cell surface of PMN when compared with elution of HLE ($p = 0.01$) or CG ($p = 0.007$). To test the release of these enzymes from the

Table I. *Free release of proteinase 3 from activated PMN*

Condition[a]	Percent Release[b] PR3	HLE	CG
Unstimulated PMN	1.6 ± 0.5[c]	1.6 ± 0.7	1.0 ± 0.4
LPS-primed and IL-8-stimulated PMN	2.2 ± 0.5	2.2 ± 0.7	1.6 ± 0.4
PAF-primed and IL-8-stimulated PMN	2.2 ± 0.5	2.2 ± 0.7	1.7 ± 0.5
TNF-α-primed and IL-8-stimulated PMN	2.7 ± 1.1	2.5 ± 1.3	2.2 ± 1.4

[a] Cells were incubated at 37°C without agonists for 30 min, or primed for 5 min with 100 ng/ml LPS or 15 min with 10^{-9} M PAF or 100 U/ml TNF-α, then stimulated for 30 min with 10^{-8} M IL-8.

[b] Release of PR3, HLE, and CG was quantified in triplicate in cell-free supernatant fluids and cell extracts of unstimulated PMN using indirect binding ELISAs. The results are expressed as a percentage of the amount of each proteinase that was present in cell extracts from unstimulated cells.

[c] Data are mean values; error bars represent SD; $n = 4$ donors.

PMN cell surface under physiologic conditions, we directly compared the release of PR3, HLE, and CG from the cell surface of activated PMN in the presence of 0.15 M NaCl at 37°C, as a function of time (Fig. 6*B*). There was significantly greater release of PR3 from the surface of activated PMN when compared with release of HLE and CG at all time points tested. Release of PR3 was detected within 5 min of exposure to isotonic buffer, and was almost complete by 15 min. In marked contrast, there was minimal release of either HLE and almost no release of CG from the surface of PMN in the presence of physiologic salt concentrations, even when the incubation period was extended to 2 h (data not shown). These data indicate that during the inflammatory response, a portion of cell surface-bound PR3 is rapidly released from the plasma membrane of activated PMN, whereas HLE and CG remain bound to the cell surface.

Discussion

PR3 is potentially an important enzyme in health and disease. However, studies of this serine proteinase have been challenging because of the lack of availability of substrates or inhibitors that are specific for PR3. In this paper, we have developed methods to circumvent some of these technical problems, permitting us to study membrane-bound PR3 on PMN with respect to regulation of expression, catalytic activity, and susceptibility to inhibition. We have demonstrated that PR3 expression is rapidly inducible on the cell surface of PMN following exposure of cells to proinflammatory mediators, under conditions in which minimal free release of PR3 can be detected. PR3 binds to the external surface of the PMN plasma membrane via a charge-dependent mechanism. Moreover, cell surface-bound PR3 on PMN is catalytically active against an important component of the extracellular matrix, yet it is substantially resistant to inhibition by physiologic proteinase inhibitors. Together, these data indicate that membrane-bound PR3 on PMN is an important extracellular, bioactive form of the enzyme in vivo that is poised to contribute in important ways to physiologic and potentially pathologic processes of PMN.

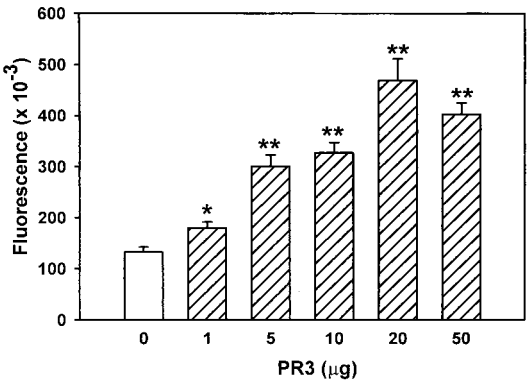

FIGURE 3. Binding of exogenous PR3 to PMN. Fixed PMN (10^6 cells) were incubated for 30 min at 4°C with varying amounts of soluble PR3 (▨) or in the absence of PR3 (□), then immunostained with rhodamine for cell surface-bound PR3. Note that soluble PR3 binds to the cell surface of fixed PMN in a dose-dependent manner. Data are mean values; error bars are SEM, $n = 100$–150 cells. *, $p = 0.004$; **, $p < 0.001$. compared with cells incubated in the absence of soluble PR3.

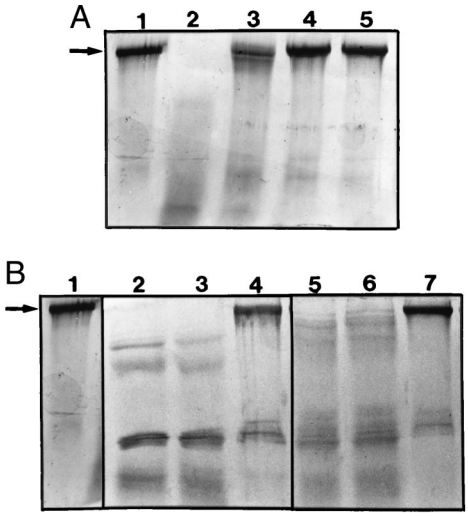

FIGURE 4. Fibronectin degradation by cell surface-bound PR3. *A*, Comparison of soluble vs membrane-bound PR3. *Lane 1*, Fibronectin alone. *Lane 2*, Fibronectin + soluble PR3. *Lane 3*, Fibronectin + exogenous PR3 bound to PMN. *Lane 4*, Control PMN incubated without exogenous PR3. *Lane 5*, Fibronectin + cell-free supernatant fluids harvested from cells that bound exogenous PR3. Note that fibronectin degradation was associated only with soluble PR3 and PMN that bound exogenous PR3. *B*, Effect of proteinase inhibitors on fibronectin degradation by soluble and membrane-bound PR3. *Lane 1*, Fibronectin alone. *Lane 2*, Fibronectin + soluble PR3. *Lane 3*, Fibronectin + soluble PR3 + SLPI. *Lane 4*, Fibronectin + PR3 + elafin. *Lane 5*, Fibronectin + exogenous PR3 bound to PMN. *Lane 6*, Fibronectin + exogenous PR3 bound to PMN + SLPI. *Lane 7*, Fibronectin + exogenous PR3 bound to PMN + elafin. Note that elafin inhibited fibronectin degradation by both soluble PR3 and membrane-bound PR3, but SLPI was ineffective against both forms of the proteinase.

Quantification of PR3 in PMN

Our results demonstrated that PMN contain ~3 pg of PR3 per cell, which is in good agreement with recently published data from Witko-Sarsat et al. (24), and it is also very similar to the quantity of HLE (1.1 pg per cell) and CG (0.85 pg per cell) contained within PMN (17). From our data, together with previously published data on the mean number of HLE-containing azurophil granules per PMN (19), and the mean volume of a single azurophil granule (20), we have calculated that each granule contains PR3 at a mean concentration of 13.4 mM.

Regulation of the expression of cell surface-bound PR3 activity

We used quantitative immunofluorescence staining to study PR3 expression on the surface of PMN, and immunogold staining confirmed that our Ab recognizes cell surface-bound PR3 rather than

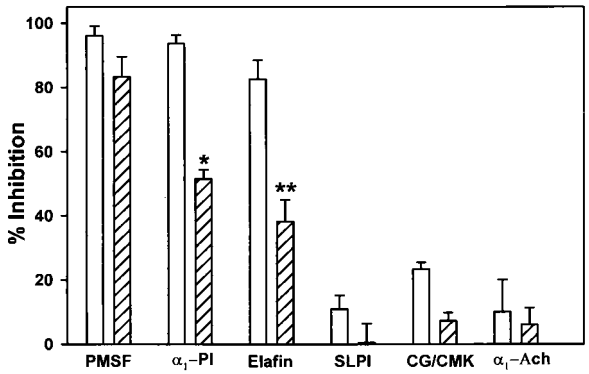

FIGURE 5. Susceptibility of membrane-bound PR3 to inhibition by proteinase inhibitors that differ in their molecular size. Soluble PR3 (□) or exogenous PR3 bound to PMN (▨) were incubated for 15 min at 25°C with or without the following inhibitors: 1) 1 mM PMSF, 2) 5 μM α_1-PI, 3) 2 μM elafin, 4) 2 μM SLPI, 5) 100 μM CG/CMK, or 6) 2 μM α_1-antichymotrypsin. Residual PR3 activity was quantified using Boc-Ala-Ala-Nva-SBzl, as described in *Materials and Methods*, and the results were expressed as percent inhibition. Note that α_1-PI, elafin, and PMSF were fully effective as inhibitors of soluble PR3. Although the low molecular mass inhibitor PMSF was substantially effective against membrane-bound PR3, the naturally occurring inhibitors α_1-PI and elafin were only partially effective against membrane-bound enzyme. The control inhibitors (SLPI, CG/CMK, and α_1-antichymotrypsin) were ineffective against either form of the proteinase. Data are mean values ± SEM; $n = 4$ experiments. *, $p = 0.002$; **, $p < 0.001$ when compared with soluble PR3 incubated with the same inhibitor.

intracellular proteins. We have shown that, on average, ~38% of unstimulated PMN from healthy donors express low levels of PR3 on their cell surface, and that cell surface expression of PR3 on PMN is readily inducible both by pharmacologic agonists and proinflammatory mediators with respect to both the mean quantity of PR3 expressed per cell and the percentage of cells that express PR3. The relatively greater effects of pharmacologic agonists on mean cell surface expression of PR3, when compared with the effects of cytokines and chemoattractants when used alone, are likely to reflect their greater effects on PMN degranulation.

Other laboratories have shown a proportion of unstimulated PMN from healthy donors express PR3 on their cell surface. Halbwachs-Mercarelli et al. (11) demonstrated that the proportion of freshly isolated PMN that expresses PR3 varies considerably between donors (mean 49.6%; range 0–95%; $n = 47$ donors), but is extremely stable for each individual over prolonged periods of time. Although we did not study our donors over time, we also found a wide range in the proportion of freshly isolated PMN from healthy individuals that express low levels of PR3 (9.5–85%). Other laboratories have shown that exposure of cells to agonists such as PMA, TNF-α, IL-8, fMLP, cytochalasin B, and TGF-β increases the proportion of PMN that express PR3 on their cell surface (2, 9–11), but the effect of these mediators on the mean quantity of cell surface-bound PR3 on the whole PMN population has not been assessed previously. It is noteworthy that one of these studies found that ~70% of PMN from patients with sepsis syndrome express PR3 on their cell surface (9). Our data suggest that exposure of PMN to cytokines and chemoattractants in vivo could mediate the observed increases in cell surface expression of PR3 on PMN during sepsis.

Although priming of cells with cytokines, followed by activation with a chemoattractant, results in striking synergistic increases in cell surface expression of PR3, minimal PR3 is freely released from cells even under these conditions. These data indicate that when cells are activated by exposure to proinflammatory mediators in vivo, PR3 bound to the cell surface of PMN is likely to be the predominant extracellular form of the enzyme.

Catalytic activity and susceptibility to inhibition of membrane-bound PR3

We are the first to report that membrane-bound PR3 on PMN is catalytically active. Earlier studies were hampered by the lack of availability of a substrate that is completely specific for PR3. The catalytic activity of endogenously expressed PR3 on the cell surface of activated PMN cannot be studied directly because activated PMN also express cell surface-bound HLE (13, 14), which has a substrate specificity that is similar to that of membrane-bound PR3. To circumvent this problem, we studied exogenous PR3 bound to unstimulated, fixed PMN. Cells that bound exogenous PR3 cleaved a synthetic oligopeptide substrate. Although it is not possible to use this substrate to quantify catalytically active PR3

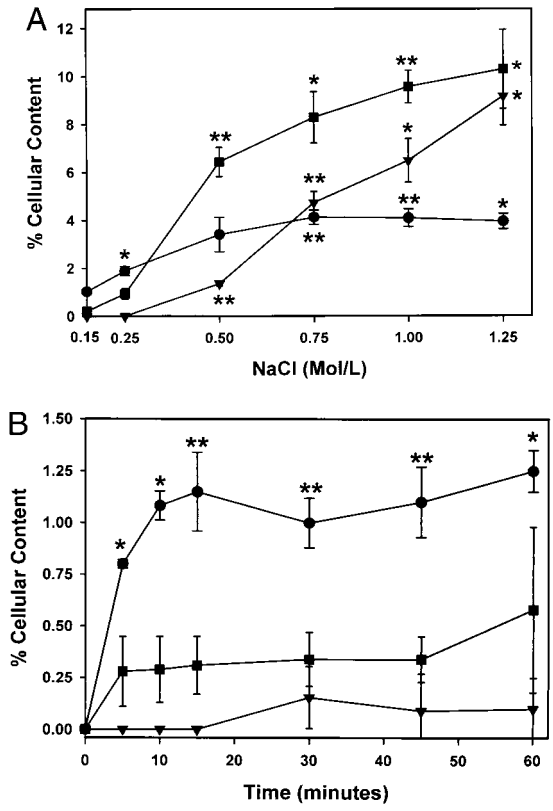

FIGURE 6. Elution of serine proteinases from the cell surface of activated PMN. PMN were optimally primed with PAF and stimulated with fMLP, then fixed to prevent leakage of intracellular proteinase, as described in *Materials and Methods*. *A*, Cells were resuspended in PBS containing 0.15–1.25 M NaCl, incubated overnight at 4°C, then cell-free supernatant fluids were assayed for immunoreactive PR3 (●), HLE (■), and CG (▲). Note that elution of PR3 was detectable in the presence of 0.15 M NaCl, and was maximal in the presence of 0.75 M NaCl. Higher concentrations of NaCl were required to elute HLE and CG from the cell surface of PMN. Data are mean values; error bars represent SEM; $n = 3$ donors. *, $p < 0.05$; **, $p < 0.01$ compared with elution of the same proteinase in the presence of 0.15 M NaCl. *B*, PMN were resuspended in PBS containing 0.15 M NaCl, incubated at 37°C for varying times, then immunoreactive PR3 (●), HLE (■), and CG (▲) and were quantified in cell-free supernatant samples. Note that release of PR3 in the presence of physiologic salt concentrations was significantly greater than that of HLE and CG at all time points tested. Data are mean values; error bars are SD; $n = 4$. *, $p < 0.03$; **, $p < 0.001$ compared with elution of HLE and CG at the same time point.

that is endogenously expressed on the surface of activated PMN, it is noteworthy that optimally activated PMN express ~12% of their cellular content of both HLE and CG on their cell surface (12, 14), and it is likely that a similar proportion of the PMN cellular content of PR3 is also expressed on the surface of optimally activated cells.

We also demonstrated that exogenous PR3 bound to PMN can degrade fibronectin, an important component of the extracellular matrix and basement membranes. These data suggest that PR3 expressed on the surface of activated PMN may play roles in the egress of cells from the vasculature and their penetration of tissues barriers, including basement membranes, as cells traverse through tissue planes en route to sites of inflammation.

We are also the first to study the susceptibility of membrane-bound PR3 on PMN to inhibition by proteinase inhibitors that vary in their molecular size. In marked contrast to soluble PR3, membrane-bound PR3 was substantially resistant to inhibition by physiologic inhibitors including α_1-PI, which is the major inhibitor of PR3 in plasma and the lower respiratory tract (25), and elafin, which may be an important inhibitor of PR3 in various secretions (26), even when these inhibitors were used at 100- to 300-fold molar excess over enzyme. The fact that the low molecular mass inhibitor, PMSF, was almost fully effective against membrane-bound PR3 indicates that steric hindrance might be the major mechanism by which membrane-bound PR3 evades inhibition by intermediate and high molecular mass inhibitors.

Mechanism of binding of HLE to the cell surface of PMN

Previous enzyme histochemical studies have demonstrated that, following stimulation of PMN, azurophil granules translocate to the cell surface and that serine proteinases become bound to the external surface of the plasma membrane near the sites of degranulation (13). Previous studies of HLE and CG have shown that the strongly cationic nature of HLE and CG enables them to bind to a negatively charged plasma membrane constituent or constituents (12, 14, 23). We tested the possibility that PR3 also binds to the cell surface by a charge-dependent mechanism, because PR3 is also a cationic proteinase (27). Our results demonstrated that elution of PR3 from the cell surface of PMN was detectable even in the presence of physiologic NaCl concentrations, and that PR3 eluted in solutions having lower ionic strength when compared with the elution of HLE, which in turn, eluted in solutions having lower ionic strength when compared with elution of CG. These data are entirely consistent with our working hypothesis, because PR3 is the least cationic, and CG is the most cationic of the three serine proteinases (isoelectric points for PR3, HLE, and CG are 9.1, 10.5, and >11, respectively (27, 28)). Detachment of PR3 from the cell surface of activated PMN is also likely to occur at sites of inflammation in vivo, and this phenomenon could be relevant to the pathogenesis of systemic necrotizing vasculitides.

We disagree with the previous findings of Witko-Sarsat et al. (2), who reported that PR3 cannot be eluted from the cell surface of human PMN. This discrepancy is likely to reflect two major technical differences between the two studies. First, Witko-Sarsat et al. studied elution of PR3 from the cell surface of unstimulated PMN, whereas we studied elution of PR3 from cells that had been activated to induce cell surface expression of PR3 on PMN following degranulation. It is possible that PR3 that is constitutively present on the cell membrane of unstimulated PMN differs in its mechanism of association with the plasma membrane when compared with PR3 which binds to the cell surface following cellular activation and degranulation. We consider this to be only a formal possibility. The second and more likely explanation for the discrepant results is that Witko-Sarsat and coworkers tested whether PR3 could be eluted from the cell surface of unstimulated PMN by exposing viable cells to nonphysiologic buffers having extreme pH (pH 3.0 and pH 10.7) and demonstrated small increases in cell surface expression of PR3. It is very likely that exposing PMN to extremes of pH adversely affects cell viability and membrane permeability. Any release of PR3 from cells stressed in this manner would mask reductions in cell surface expression of PR3 induced by exposure of PMN to highly acidic or basic pH. Witko-Sarsat and coworkers did not test the effects of their treatments on cell viability or free release of PR3, nor did they study the effects of extreme pH on elution of HLE and CG (as controls), which have been shown to bind via ionic interactions to the cell membrane of PMN (12, 14, 25). In the current study, we exposed activated PMN to solutions having increasing ionic strength after fixing the cells to prevent release of intracellular proteinases. With this experimental design, we were able to demonstrate elution of PR3, HLE, and CG from the cell surface in an order that was related to their isoelectric points. We have also confirmed that there is no significant release of LDH activity from PMN during this assay. We are thus confident that PR3 can be eluted from the cell surface of PMN (and that it is released from the cell surface even under physiologic conditions) and that ionic interactions are important in the binding of this proteinase to the cell membrane following cellular activation.

Our data indicate that the binding of serine proteinases to the cell membrane may be a mechanism by which cells preserve and restrict the catalytic activity of their serine proteinases to the pericellular environment, and thereby ensure controlled extracellular proteolysis during physiologic processes. A potentially important difference between PR3 and the other two serine proteinases, however, is that PR3 detaches more readily from the surface of PMN when compared with HLE or CG, and PR3 could thus become exposed to immune-competent cells that are some distance removed. This process might lead to the greater propensity for genetically susceptible individuals to generate ANCA directed against PR3, as opposed to HLE or CG.

The molecule(s) on the PMN plasma membrane to which PR3 binds remain unknown. However, ongoing studies in our laboratory indicate that PMN express common, low affinity, yet high-volume binding sites for HLE and CG (C.A.O. and E.J.C., unpublished observations). In addition, treatment of PMN with chondroitinase partially inhibits the binding of HLE and CG to PMN indicating that binding of these serine proteinases to PMN is mediated, in part, by chondroitin sulfate-containing proteoglycans. We have also shown that excess unlabeled PR3 can compete with labeled HLE or CG for binding to the plasma membrane indicating that PR3 binds to sites with similar characteristics (C.A.O. and E.J.C., unpublished observations). Low affinity, high-volume binding sites would be ideally suited for binding the mM concentrations of PR3 that are transiently present near the cell surface of PMN following degranulation. Other biologically important mediators have also been shown to bind to negatively charged proteoglycans on cell surfaces. For example, platelet factor 4 has been shown to bind to chondroitin sulfate proteoglycans on the cell surface of PMN (29). In addition, heparan sulfate proteoglycans on the cell surface of endothelial cells and fibroblasts serve as coreceptors for basic fibroblast growth factor, which protect basic fibroblast growth factor from proteolytic degradation (30–32). Cell surface proteoglycans may thus serve to focus, regulate and preserve the activity of diverse bioactive molecules on inflammatory cells and resident cells in vivo.

Conclusions

PR3 that is bound to the cell surface of PMN retains its activity even in the presence of physiologic inhibitors, and may thereby play important roles in the physiologic processes of PMN. The

binding of PR3 to the surface of inflammatory cells serves to focus, preserve, and restrict the catalytic activity of this proteinase to the immediate pericellular environment. Because membrane-bound PR3 can degrade large polypeptide substrates, such as fibronectin, this form of the proteinase may play a role in the penetration of tissue barriers by PMN as they migrate through tissues. Moreover, the observations that soluble PR3 can also activate precursor forms of cytokines such as TNF-α, IL-8, and IL-1β (7, 8) suggest the possibility that catalytically active PR3 expressed on the cell surface of PMN may also participate in cytokine processing, and thereby play a role in regulating inflammation.

Rapidly inducible expression of PR3 on the cell surface of PMN in healthy individuals may be a normal phenomenon during the inflammatory response, and cell surface-bound PR3 may be the predominant extracellular form of the proteinase when cells are activated by proinflammatory mediators. However, in susceptible individuals, persistent expression of PR3 on the cell surface of inflammatory cells and/or detachment of PR3 from the surface of activated cells may lead to the generation of autoantibodies, which in turn activate more PMN and contribute to self-perpetuating tissue injury in systemic necrotizing vasculitis syndromes.

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