Involvement of a metalloprotease in the shedding of human neutrophil FcγRIIIB

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Abstract FcγRIIIb is a glycosylphosphatidylinositol(GPI)-anchored, low-affinity IgG receptor, expressed exclusively on human neutrophils. Upon activation or apoptosis of neutrophils, FcγRIIIb is shed from the cell surface, but the enzyme(s) responsible for this process is (are) still unknown. Recently, metalloproteases have been suggested to mediate the shedding of cell surface proteins such as L-selectin and TNF-α. Using hydroxamic acid-based inhibitors of this class of proteases (BB-3103, Ro31-9790), we have observed a clear inhibitory effect on FcγRIIIb shedding after PMA stimulation of neutrophils or induction of apoptosis. These inhibitors did not affect PMA-induced degranulation or superoxide generation.

Key words: FcγRIIIb; Proteolytic cleavage; Hydroxamic acid-based inhibitor; Metalloprotease; Apoptosis

1. Introduction

Human neutrophils express two receptors for IgG: FcγRIIa (CD32) and FcγIIIb (CD16). FcγRIIa is a transmembrane protein, whereas FcγRIIIb is linked to the membrane via a glycosylphosphatidylinositol(GPI)-anchor [1]. Upon activation of neutrophils [2] or during apoptosis [3,4], FcγRIIIb is shed from the cell surface by proteolytic cleavage, but the enzyme(s) responsible for this process is (are) still unknown. Earlier studies indicated that the release of FcγRIIIb could be inhibited by protease inhibitors of serine proteases and metalloproteases [5,6]. Likewise, several other membrane molecules, such as L-selectin and TNF-α are released by proteolytic events from the leukocyte surface upon activation (see [7] for review).

Soluble FcγRIII (sFcγRIII) in vivo is found in saliva, synovial and seminal fluid, serum and plasma [8,9]. Also FcγRIIIa-bearing cells, such as macrophages and NK cells, release this receptor by a proteolytic event. Plasma sFcγRIII, however, is mainly derived from neutrophils (FcγIIIb) and is related to the production of neutrophils by the bone marrow [10–12].

The biological role of sFcγRIIIb is still unclear. Soluble FcγIII is still able to bind IgG, as shown by precipitates from plasma [5]. Studies with recombinant sFcγRIIIb showed binding to a subpopulation of B cells, T cells and monocytes [13], and inhibitory effects on B cell proliferation and IgG/IgM production. Studies with purified sFcγRIIIb from serum have demonstrated cell activation through interaction with complement receptors (CR3 and CR4) [14]. These studies indicate that sFcγRIIIb plays an important role in Fc-dependent immune responses.

Recently, a new class of compounds has been developed to inhibit matrix metalloproteases (MMP), hydroxamic acid-based inhibitors, which also block other zinc-dependent proteases such as mammalian adamalysins (ADAMs). Shedding of a range of cell surface molecules, such as L-selectin, TNF-α, Fas ligand, Il-6R and TNFR's, is blocked by these compounds[7].

In the present study we investigated by means of hydroxamic acid-based inhibitors the possible involvement of metalloproteases in the shedding process of FcγRIIIb.

2. Materials and methods

2.1. Materials

PMA and cytochalasin B were obtained from Sigma Chemical Co., St. Louis, MO, USA. Ro31-9790 was a kind gift from Roche Discovery, Welwyn Garden City, UK and BB-3103 was a kind gift of British Biotechnology Pharmaceuticals, Oxford, UK. The following monoclonals were obtained from our own institute: CLB-Fcgran1 (FcγRIII), fluoresceine-isothiocyanate (FITC)-labeled CLB-Fcgran1, B13.9 (CD66b) and irrelevant murine control IgG1. Leu-8 (CD62-L) was obtained from Becton and Dickinson, San Jose, CA, USA. (FITC)-labeled Annexin V was a kind gift of Dr Reutelingsperger, Biochemistry Department, University of Limburg, Maastricht, The Netherlands. The inhibitory MoAb REGA-3G12 against human neutrophil gelatinase B (MMP-9)[15] was a kind gift of Dr. Liesbet Paemen, Laboratory of Molecular Immunology, Rega Institute for Medical Research, Leuven, Belgium.

2.2. Isolation of neutrophils

Peripheral blood was obtained from healthy volunteers. Granulocytes were purified from the buffy coats of 500 ml of blood anticoagulated with 0.4% (w/v) trisodium citrate, as described before [16]. In short, mononuclear cells and platelets were removed by density centrifugation over isotonic Percoll with a specific gravity of 1.076 g/ml. Erythrocytes were removed by a 10-min treatment with ice-cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). The remaining granulocytes were washed twice in phosphate-buffered saline (PBS) and were resuspended in incubation medium [132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM $NaHPO_4$, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human serum albumin (pH 7.4)] at a concentration of 10^7 cells/ml. The purity and viability of the neutrophils was over 95%.

2.3. Preparation of cytoplasts

Enucleated neutrophils were prepared as described before [17]. In short, neutrophils were suspended in 12.5% (w/v) Ficoll solution with 20 μM cytochalasin B. This cell suspension (approximately 10^8/ml) was preincubated for 5 min at 37°C. The suspension (10 ml) was then layered on a prewarmed (2 h at 37°C), discontinuous density gradient (10 ml of 16% Ficoll on top of 10 ml of 25% Ficoll). Cytochalasin B (20 μM) was present throughout the gradient. Polycarbonate centrifuge tubes (2.5×8.9 cm; Beckman Instruments Inc., Palo Alto, CA, USA) were used. After loading of the cells, the gradients were centrifuged for 30 min at 81 000×*g* and 33°C in an ultracentri-

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fuge (Kontron Electronic, Inc., Ultracentrifuge TGA 50, Zürich, Switzerland), in a SW27 swing-out rotor (Beckman Instruments, Inc.) prewarmed for 4 h at 37°C. Cytoplasts were harvested from the 12.5%/16% interface and were washed twice with incubation medium.

2.4. Cell activation

Neutrophils (10^7/ml) or cytoplasts (4×10^7/ml) in incubation medium were incubated in a shaking waterbath for 10 min at 37°C with the amount of Ro-31-9790 or BB-3103 indicated in the figures. The cells were subsequently activated with PMA (100 ng/ml) for 10 min at 37°C. Cell-free supernatants were collected and cell pellets were fixed in ice-cold fixation buffer [5PBS containing bovine serum albumin (0.3% w/v) and paraformaldehyde (1% w/v)] for 10 min on ice.

2.5. Flow cytometry

Expression of FcγRIIIb, L-selectin and CD66b of the neutrophils or cytoplasts were determined by FACScan analysis. Fixed neutrophils or cytoplasts were washed once and were then incubated with MoAb for 45 min at 4°C in PBS containing bovine serum albumin (0.3% w/v). The cells were subsequently stained with fluoresceine-isothiocyanate (FITC)-labeled goat-anti-mouse-Ig for 30 min at RT. After washing, the cells were resuspended in PBS containing bovine serum albumin (0.3% w/v) and fluorescence was measured (FACScan, Becton and Dickinson, San Jose, CA, USA).

2.6. Measurement of soluble FcγRIIIb in cell supernatants

Soluble FcγRIIIb in neutrophil supernatant samples was measured by ELISA as described by Koene et al. [18]. In short, 96-well ELISA plates were coated with an FcγIIIb catching MoAb CLB-Fcgran1 and detection of sFcγRIIIb in the samples was performed with a biotin-labeled, polyclonal rabbit-anti-human-FcγRIIIb antibody. After addition of streptavidin poly-horse-radish-peroxidase and substrate buffer, the color reaction was allowed to proceed for about 15 min after which the absorbance at 450 nm was measured in a Titertek multiscan ELISA reader (Flow Laboratory, Rockville, MD, USA). The concentration of sFcγIIIb in each sample was calculated from a standard curve obtained by serial dilutions of human plasma containing 5 nM sFcγIIIb [5].

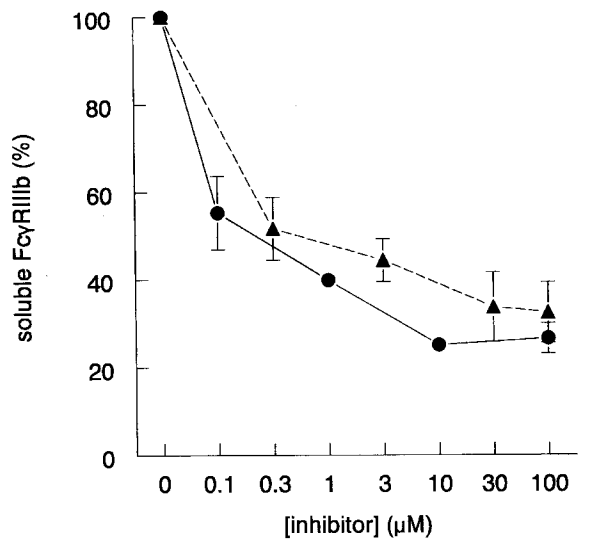

Fig. 1. Inhibition of PMA-induced FcγRIIIb shedding by hydroxamic acid-based inhibitors measured by ELISA. Human neutrophils (10^7/ml) were preincubated with different concentrations of BB-3103 (●) or Ro31-9790 (▲) for 10 min. After 10 min of PMA activation supernatant was collected and soluble FcγRIIIb was measured by ELISA. The concentration of sFcγRIII in the absence of inhibitors was taken as 100%. This concentration amounted to 0.87 ± 0.25 ($n=5$) pmol/ml. The results shown represent the mean of percentage soluble FcγIIIb ± S.D. of three independent experiments.

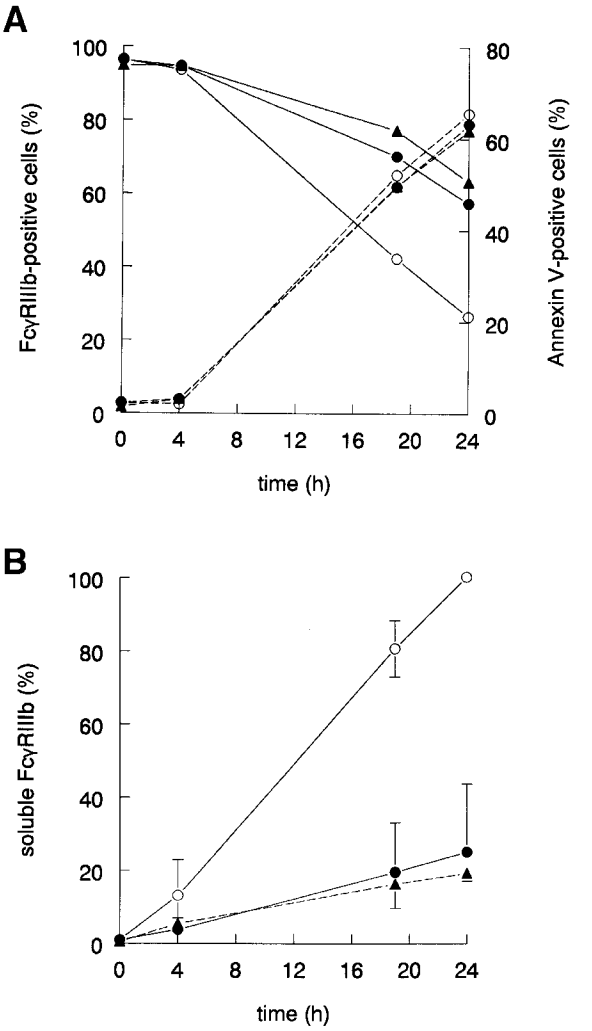

Fig. 2. (A) Flow cytometry analysis of FcγRIIIb expression and Annexin V binding during apoptosis in presence of hydroxamic acid-based inhibitors. Human neutrophils (10^7/ml) were incubated for 24 h in the presence of DMSO (0.3%) as control (○), with BB-3103 (100 μM, ●) or with Ro31-9790 (100 μM, ▲). At various times of incubation, cells were analyzed for FcγRIIIb expression (solid lines) and Annexin V binding (dashed lines). The results represent the mean of three independent experiments. (B) Inhibition of FcγRIIIb shedding by hydroxamic acid-based inhibitors during apoptosis as measured by ELISA. Human neutrophils (10^7/ml) were incubated for 24 h in the presence of DMSO (0.3%) as control (○), with BB-3103 (100 μM, ●) or with Ro31-9790 (100 μM, ▲). Soluble FcγRIII levels in cell-free supernatants at different times of incubation are shown. The concentration of sFcγRIII in supernatants after 24 h in the absence of inhibitors was taken as 100%. This concentration amounted to 0.62 ± 0.11 pmol/ml ($n=3$). The results represent the mean of three independent experiments.

2.7. Apoptosis

Neutrophils (10^7/ml) in medium (Iscove's with 10% (v/v) fetal calf serum) were cultured for 24 h at 37°C in 96-well plates in the presence of 100 μM BB-3103 or Ro31-9790. At various times, cell samples were taken. Cell-free supernatant was collected for measurement by ELISA of sFcγIIIb content. The cell pellets were washed and resuspended in ice-cold PBS containing bovine serum albumin (0.3% w/v) and were stained with CD16-FITC for 30 min at 4°C. After washing, the cells were resuspended in ice-cold PBS containing bovine serum albumin (0.3% w/v), and the fluorescence was measured (FACScan, Becton and Dickinson, San Jose, CA, USA). For Annexin V binding, incubation with FITC-labeled Annexin V and washing was performed in ice-cold incubation buffer [4].

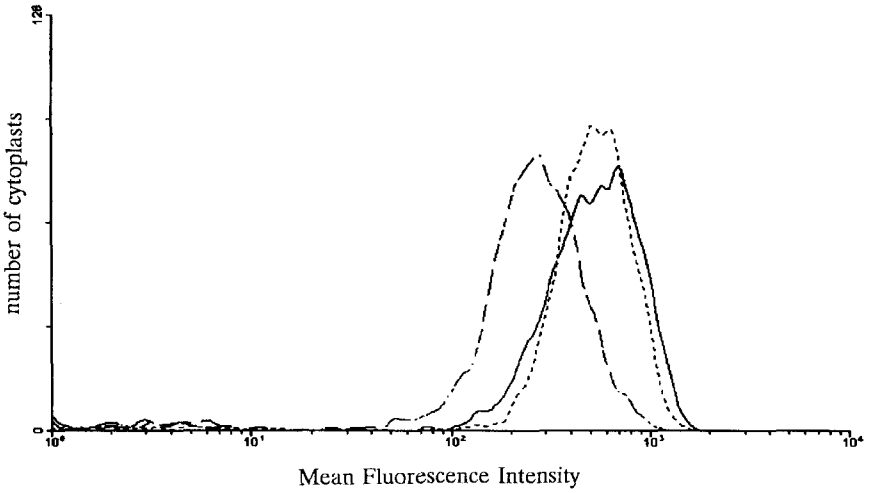

Fig. 3. Inhibition of PMA-induced FcγRIIIb shedding from cytoplasts by BB-3103. Cytoplasts (4×10^7/ml) were preincubated with BB-3103 (100 μM) for 10 min. After 10 min of PMA activation, the cytoplasts were fixed and analysed for FcγRIIIb expression by flow cytometry. Depicted in the figure are: unstimulated cytoplasts (solid line), PMA-treated cytoplasts (broken line) and BB-3103/PMA-treated cytoplasts (dotted line).

3. Results

After PMA activation, neutrophil FcγRIIIb expression measured by flow cytometry was approximately 30% of the unstimulated control. In preliminary experiments, the hydroxamic acid-based inhibitors Ro31-9790 and BB-3103 inhibited this decrease in FcγRIIIb expression for about 50% (results not shown). The expression measured in this way is the net result of shedding from the surface and upregulation from secretory vesicles containing FcγRIIIb [11,19]. The extent of inhibition of FcγRIIIb shedding can be more reliably estimated by measuring soluble FcγRIIIb in the neutrophil supernatants. An inhibitory effect up to 70% on FcγRIII shedding was measured with IC_{50}'s of approximately 0.1 μM for BB-3103 and 0.2 μM for Ro31-9790 (Fig. 1). Previous experiments with serine- and metalloprotease inhibitors such as DFP, PMSF, EDTA and 1,10-phenanthroline in our group and others [5,6] already indicated that serine- and metalloproteases are probably involved in the shedding process of FcγRIIIb. However, in our hands these compounds inhibit not only the shedding process but also other processes, such as the fusion of granules with the cell surface and the oxidative burst. The presence of hydroxamic acid-based inhibitors did not result in such inhibition. For instance, the PMA-upregulated expression of CD66b [20] was not affected: after FACS analysis values of 928 ± 429 (Mean Fluorescence Intensity) for controls and 922 ± 311 ($n=3$) in the presence of 10 μM BB-3103, were found. Superoxide generation under these conditions amounted to in 7.8 ± 0.5 nmol O_2^-/min/10^6 cells (mean ± S.E.M. of 4 experiments) for the controls, 8.5 ± 0.7 (10 μM BB-3103) and 7.2 ± 0.7 (10 μM Ro31-9790).

Also during apoptosis, FcγRIIIb shedding was inhibited by the hydroxamic acid-based inhibitors (Fig. 2). Apoptosis itself, as measured by Annexin V binding [4], was not affected by these inhibitors. These results indicate that a similar proteolytic enzyme is active during PMA activation and during apoptosis in cleaving the FcγRIIIb molecule from the surface of the human neutrophil.

Human neutrophils contain two matrix metalloproteases in their specific granules, collagenase (MMP-8) and gelatinase B (MMP-9). To investigate their involvement in the FcγRIIIb shedding process, two experimental approaches were used. First, cytoplasts, which are enucleated neutrophils devoid of granules [17], were prepared. Cytoplasts retain functional responses such as respiratory burst, phagocytosis and shedding of FcγRIIIb [5,17]. In the presence of the hydroxamic based inhibitor BB-3103 (100 μM), no downregulation of FcγRIIIb was observed after PMA activation (Fig. 3). Similar results were obtained with Ro31-9790 (results not shown). This indicates that the target protease for these inhibitors is still present in neutrophil cytoplasts and thus may be membrane bound. However, secretion of collagenase and gelatinase B and rebinding of these enzymes to the cell surface during cytoplast preparation cannot be excluded. We therefore used

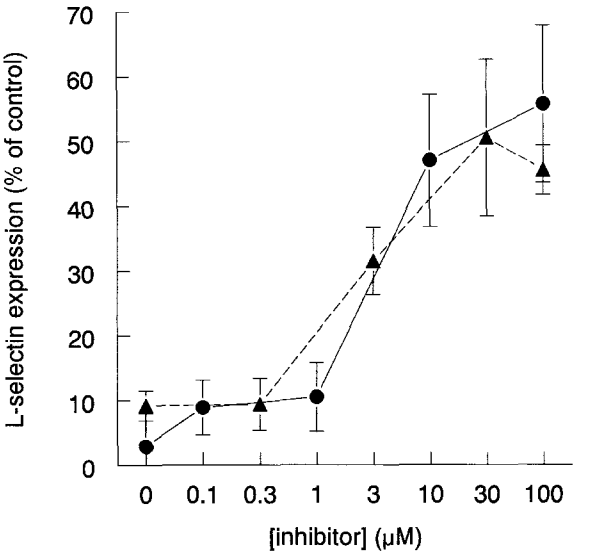

Fig. 4. Inhibition of PMA-induced L-selectin shedding by hydroxamic acid-based inhibitors. Human neutrophils (10^7/ml) were preincubated with different concentrations of BB-3103 (●) or Ro31-9790 (▲) for 10 min. After 10 min of PMA activation, the cells were fixed and analyzed for L-selectin expression. The results shown represent the mean ± S.D. of three independent experiments.

an inhibitory MoAb against human neutrophil gelatinase B [15] up to 50 μg/ml, and found no inhibitory effect on FcγRIIIb shedding as measured by FACS analysis: Mean Fluorescence Intensities amounting to 1684 ± 202 (control) and 1733 ± 165 (REGA-3G12) $n = 3$, were observed. Gelatinase B is therefore unlikely to be the shedding protease of FcγRIIIb, as discussed before [6].

Other membrane-bound molecules that can be shed after stimulation from human neutrophils are sialophorin (CD43), CD44, and L-selectin (CD62-L) [6,7]. Previous studies with leukocytes have shown that L-selectin shedding can be blocked by hydroxamic acid-based inhibitors [21–24]. In the present study, we have determined the sensitivity of the downregulation of L-selectin for these inhibitors under the same experimental conditions as those used for FcγRIIIb shedding (Fig. 1). It appears that the IC_{50} of these inhibitors for FcγRIIIb downregulation is significantly lower than for L-selectin downregulation (Fig. 4). For the latter experiment IC_{50}'s of approximately 3 μM were found, which is in good agreement with data published by Allport et al. [25]. An explanation could be that shedding of L-selectin requires another enzymatic process than FcγRIIIb shedding. Another observation that supports this explanation is the kinetics of downregulation of both molecules: shedding of L-selectin is much faster after PMA activation than FcγRIIIb shedding (results not shown).

4. Discussion

In the present study the involvement of a metalloprotease in the shedding of human neutrophil FcγRIIIb was investigated. A clear inhibitory effect was observed with hydroxamic acid-based inhibitors. The presence of the inhibitors is required throughout the experiments (results not shown), which suggests that the metalloprotease(s) responsible for FcγRIIIb shedding is upregulated from granules and/or activated at the cell surface. Involvement in the shedding process of known matrix metalloproteases from the granules of the neutrophil, collagenase and gelatinase B, is unlikely on the basis of our cytoplast experiments. Also secretion and rebinding of gelatinase B, after which cleavage could occur, is very unlikely, because inhibition of this enzyme had no effect. Because FcγRIIIb is a GPI-anchored molecule, also phospholipases could be candidate for the shedding of FcγRIIIb. Earlier studies, however, have excluded this possibility [5]. A membrane-bound protease is more likely to be involved in the cleavage of FcγIIIb.

Hydroxamic acid-based inhibitors such as Ro31-9790 and BB-3103 have originally been developed to inhibit matrix metalloproteases. Ro31-9790 is known to be a potent inhibitor of fibroblast collagenase [22], whereas BB-3103, a water-soluble analogue of batimastat, shows inhibitory activity against both fibroblast collagenase and stromelysin 1 [26]. A possible target for these inhibitors could be a member of the family of membrane-bound MMP's which has recently been cloned. Of these, expression of MT4-MMP has been shown on human neutrophils [27]. However, TACE (TNF-α Converting Enzyme) is also inhibited by hydroxamic acid-based inhibitors but is not a matrix metalloprotease [28]. Recent cloning of TACE has shown that this enzyme is a metalloprotease disintegrin that cleaves TNF-α from cells [29,30]. This enzyme belongs to the family of ADAMs, which are thought to play an important role in cell-cell and cell-matrix interactions [31]. Also ADAM-10, another enzyme belonging to this family has shown to have TACE activity [32]. If this class of enzymes is responsible for the cleavage of all surface molecules, three possible candidates (other than MT4-MMP mentioned above) can be envisaged to cleave L-selectin and/or FcγRIIIb: the recently discovered TACE, CD156 (ADAM-8) [33] and possibly ADAM-10. A comparison of kinetics of shedding and of sensitivity towards metalloprotease inhibitors suggests that FcγRIIIb shedding is probably mediated by other enzymes than those involved in L-selectin cleavage. Further studies are needed to identify the protease(s) responsible for the shedding of FcγRIIIb.

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