Differences between human proteinase 3 and neutrophil elastase and their murine homologues are relevant for murine model experiments

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Abstract Direct comparisons of human (h) and murine (m) neutrophil elastase (NE) and proteinase 3 (PR3) are important for the understanding and interpretation of inflammatory and PR3-related autoimmune processes investigated in wild-type-, mNE- and mPR3/mNE knockout mice. To this end, we purified recombinant mPR3 and mNE expressed in HMC1 and 293 cells and compared their biophysical properties, proteolytic activities and susceptibility to inhibitors with those of their human homologues, hPR3 and hNE. Significant species differences in physico-chemical properties, substrate specificities and enzyme kinetics towards synthetic peptide substrates, oxidized insulin B chain, and fibrinogen were detected. MeOSuc-AAPV-pNA and Suc-AAPV-pNA were hydrolyzed more efficiently by mPR3 than hPR3, but enzymatic activities of mNE and hNE were very similar. Fibrinogen was cleaved much more efficiently by mPR3 than by hPR3. All four proteases were inhibited by α_1-antitrypsin and elafin. Eglin C inihibited mNE, hNE, mPR3, but not hPR3. SLPI inhibited both NEs, but neither PR3. The custom-designed hNE inhibitor, Val_{15}-aprotinin, is a poor inhibitor for mNE. In conclusion, appropriate interpretation of experiments in murine models requires individual species-specific assessment of neutrophil protease function and inhibition.

Keywords: Proteinase 3; Neutrophil elastase; Neutrophil serine protease; ANCA; Species differences

1. Introduction

Human neutrophil elastase (hNE, EC 3.4.21.37) and proteinase 3 (hPR3, EC 3.4.21.76) are neutral serine proteases stored in granules of neutrophils and monocytes. They share 55% amino acid homology and many structural and functional characteristics [1–3].

HNE has been studied for decades because of its pathophysiologic role in diseases ranging from acute lung injury and emphysema to cyclic neutropenia [4,5]. More recently, interest in hPR3 has arisen because it is the target antigen for specific, seemingly pathogenic autoantibodies anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis [6–8], and because an HLA-A2.1-restricted hPR3-derived nonamer has been identified as a leukemia-associated antigen with therapeutic potential [9].

Despite similarities in substrate specificity and inhibitor spectrum, significant functional differences between the two proteases become recognized [10]. Insights about the biological functions of neutrophil serine proteases and their contributions to disease in vivo are increasingly extrapolated from targeted gene knock-out mice [11–15]. However, the functional characteristics of the murine neutrophil serine proteases have not yet been studied. Several lines of evidence suggest that biologically relevant functional differences between the proteases from different species should be anticipated. For instance, human and rodent α-chymases have 72% sequence homology. Yet, the rodent isozymes do not behave chymotrypsin-like as human α-chymases do, but are elastase-like proteases [16]. Different substrate specificities and possible biological functions are also suggested by the different protease inhibitor-repertoire of mice [17–19]. Consequently, more detailed knowledge about the functional similarities and dissimilarities of murine neutrophil elastase (mNE) and proteinase 3 (mPR3) compared to their human homologues is necessary for the appropriate interpretation of mouse model experiments.

This is the first study to evaluate substrate specificities and inhibitor profiles of mPR3 and mNE. Until now, such studies have been limited by the availability of purified mPR3 and mNE. Our findings indicate that physico-chemical properties and functional characteristics of hPR3 and mPR3 differ substantially. Despite more significant functional similarities between hNE and mNE, custom-designed inhibitors for hNE may be poor inhibitors for mNE. Therefore, individual functions and inhibitors need to be evaluated in a species-specific manner.

2. Materials and methods

2.1. Materials

All reagents were from Sigma (St. Louis, MO), unless specified otherwise. Elafin was from Peptides International, Inc. (Louisville, KY), Secretory Leukocyte Protease Inhibitor (SLPI) from R&D Systems (Minneapolis, MN), purified human neutrophil PR3 (PMN-hPR3) and elastase (PMN-hNE) from Athens Research and

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Abbreviations: ANCA, anti-neutrophil cytoplasmic antibodies; HMC-1, human mast cell line 1; NE, neutrophil elastase; PR3, proteinase 3; h, human; m, murine; MeO, methoxy; Suc, succinyl; pNa, paranitroanilide; SLPI, secretory leukoprotease inhibitor

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Technology (Athens, GA). The inhibitor Val_{15}-aprotinin was a kind gift from Prof. Gordon Lowe, Dyson Perris Laboratory, Oxford, UK [20]. The human mast cell line, HMC-1, was a kind gift from Dr. Joseph Butterfield, Mayo Clinic, Rochester, MN; the human kidney epithelial cell line, 293, were purchased from the American Type Tissue Culture.

2.2. Expression of recombinant serine proteases

The cDNA constructs used are shown in Fig. 1A. Generation of wild-type hPR3 and mPR3 cDNA, corresponding expression plasmids, and transfection into HMC-1 cells have been described [21,22]. The mPR3 cDNA was amplified from total bone marrow cDNA using the primers DJ398 (5′-CCAGGTACCTGTCCATGGCTG-GAAGCT-3′) and DJ427 (5′-AAGGGTACCTCAGGGCCCTGC-3′). The amplified product subcloned into the *Kpn*I restriction site of a pcDNA3.1/Zeo vector (Invitrogen). The resulting plasmid was linearized with *Apa*I at the 3′ end of the mPR3 insert, and the coding sequence for a cmyc-poly-His-tag was added. This sequence was derived from a pPICZ vector by digestion with *Apa*I and *Bam*HI. Before ligation into the *Apa*I site, the *Bam*HI was filled in to generate two blunt ends and to reconstitute the *Bam*HI site of the pcDNA3.1 expression plasmid.

The cDNA construct hPR3-cmyc coding for full-length hPR3 with a carboxy-terminal cmyc-poly-His-tag was transfected into 293 cells [23].

The mNE cDNA was similarly amplified from total bone marrow cDNA using the primers DJ566 (5′-GGGGATCCGCGAGAACA-CAGCC-3′) and DJ 567 (5′-GGGGTGGGAGTGCAGACAGGT-3′). The PCR product was digested with *Bam*HI.

The cDNA construct mNE-cmyc codes for full-length mNE carrying the carboxy-terminal cmyc-poly-His-tag. It was generated by adding HindIII and XbaI restriction sites to the cDNA coding for full-length mNE using primers OW1 (5′-AAGCTTGGTACCGAGCTCG-GATCC-3′) and OW2 (5′-TCTAGAGTTGG-TCCTGCCCTC-3′). The resulting insert was cloned into the *Hind*III/*Xba* I-restricted expression vector pcDNA4/Myc-HisA (Invitrogen). The nucleotide sequence for mNE was identical to the published sequence, XM_109573 (GenBank). The plasmid was transfected into 293 cells, and expressing 293/mNE-cmyc cell clones were selected using Zeocin (Gibco, Grand Island, NY, USA) [24].

2.3. Purification of recombinant hPR3 and mPR3 from HMC-1 cells

Recombinant hPR3 was expressed in HMC-1 cells [21]. HMC-1/hPR3 cells were lysed by repeated freezing and thawing followed by incubation in 0.02 M Tris–HCl, 0.15 M NaCl, 1% Triton X-100, pH 7.4. After centrifugation, the supernatant was diluted 1:3 with water, extracted with Accell QMA (water, 0.3 mg/ml) and centrifuged. The supernatant was adjusted to pH 5.5 and applied to a Mono S column (Pharmacia) equilibrated in 0.05 M NaOAc, pH 5.5. Proteins were eluted with a linear gradient from 0 to 3 M NaCl and then with 0.05 M NaOAc, 1 M NaCl, 0.1% Triton X-100, pH 5.5 (Fig. 2A). Immunoreactivity [25] and hydrolytic activity were monitored throughout the various steps of the purification. A significant portion of bound hPR3 was eluted around 1.5 M NaCl (hPR3-salt). The peak of immunoreactivity eluted first and displayed little hydrolytic activity suggesting that these fractions contain a large proportion of incompletely processed pro-hPR3. A significant proportion of hPR3 could only be eluted with 0.1% Triton X-100 (hPR3-Tx100). The SDS-electrophoretic migration patterns of hPR3-salt and hPR3-Tx100 were similar (Fig. 2A). However, the lowest molecular mass band (most highly processed) was more prominent in the hPR3-Tx100 pool. Both, purified hPR3-salt and hPR3-Tx-100 were dialyzed against 0.05 M MES, 0.7 M NaCl, pH 4.5 (same as the PMN-hPR3 and PMN-hNE buffer) and stored at −20 °C in plastic tubes until use.

Recombinant mPR3 was expressed in HMC-1 cells [22]. After gentle washing in PBS, HMC-1/mPR3 cells were suspended in 10 ml of PBS, fixed with 40 ml of cold 95% ethanol and stored at 4 °C overnight. After centrifugation the pellet was brought up in PBS, centrifuged, and the supernatant was subjected to sequential 40% and 55% ethanol-precipitation. The supernatant was then subjected to 80% ethanol-precipitation. The resulting precipitate was dissolved in 0.01 M

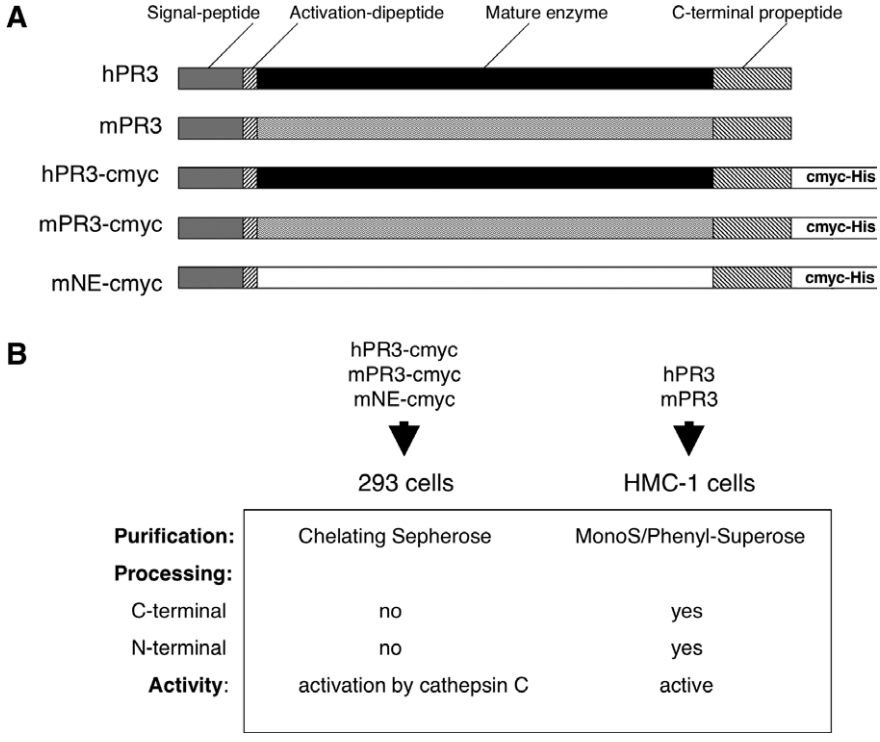

Fig. 1. Expression of recombinant human and murine neutropil serine proteases. (A) Schematic diagram of the cDNA constructs used. The signal peptide, amino-terminal activation-dipeptide, mature enzyme, and carboxy-terminal propeptide sequences are indicated. (B) Plasmids containing the cDNA constructs coding for wild-type hPR3 and mPR3 were transfected into HMC-1 cells. The expressed recombinant hPR3 and mPR3 were purified from HMC-1/hPR3 and HMC-1/mPR3 cells. The cDNA constructs hPR3-cmyc, mPR3-cmyc, and mNE-cmyc code for the full length pro-enzymes carrying a carboxy-terminal cmyc-poly-His-tag. They were transfected into 293 cells. Using the carboxy-terminal cmyc-poly-His-tag, the pro-enzymes were purified from the culture supernatant and activated by amino-terminal cleavage of the activation-dipeptide with bovine cathepsin C.

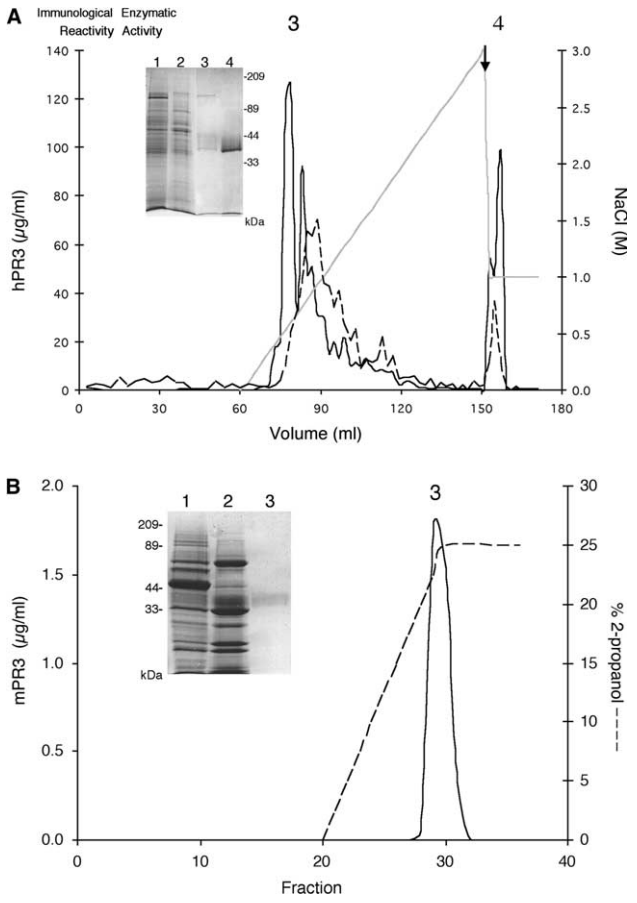

Fig. 2. Purification of human and murine recombinant PR3 from HMC-1 cells. (A) Recombinant hPR3. Proteins from HMC-1/hPR3 cell lysate and not bound to an anion exchanger were applied to a Mono S column and eluted with (1) a linear gradient from 0 to 3 M NaCl, and (2) 0.05 M NaOAc, 1 M NaCl, 0.1% TritonX-100, pH 5.5 (arrow). The hPR3 concentrations (dashed line) were determined by hydrolysis of MeOSuc-AAPV-pNA and based on active site titration data. Immunologically reactive hPR3 was quantified by capture ELISA (solid line) [25]. The inset shows SDS–PAGE (12% gel) of TCA-precipitated samples taken at different stages of purification. Lanes 1 and 2 represent 20 μg total protein of the cell lysate and QMA supernatant, respectively. Lanes 3 and 4 represent 5 μg of total protein from the corresponding indicated elution peaks. (B) Recominant mPR3. The phenyl-superose column was equilibrated in 0.3 M NaOAc pH 5.5, loaded with prepared sample and eluted with (1) a linear gradient to 0.05 M NaOAc, pH 5.5 and (2) a linear gradient to 25% 2-propanol. The mPR3 concentrations (solid line) were determined by hydrolysis of MeOSuc-AAPV-pNA and based on active site titration data. Insrt: Cell lysate containing 1 μg of active mPR3 (lane 1), Mono Q flow through containing 2 μg of active mPR3 (lane 2), and purified material from the Phenyl-Superose elution peak (3) containing 2 μg of active mPR3 (lane 3), were TCA-precipitated and separated by SDS–PAGE (12% gel).

Tris–HCl, 0.075 M NaCl, pH 8 and applied to a Mono Q column (Pharmacia) equilibrated with the same buffer. The flow-through was again subjected to 80% ethanol-precipitation. The precipitate was dissolved in 0.02 M Tris–HCl, 0.15 M NaCl, pH 7.4, to which an equal volume of 0.3 M NaOAc, pH 5.5 was added. After centrifugation the supernatant was applied to a phenyl-superose column equilibrated with 0.3 M NaOAc, pH 5.5 and subjected to a linear gradient to 0.05 M NaOAc, pH 5.5. The mPR3 was eluted with a linear gradient to 50% 2-propanol in 0.05 M NaOAc, pH 5.5 (1:1, v/v) (Fig. 2B), and stored in the elution buffer at 4 °C in glass tubes until use. Amino-terminal sequencing of the predominant 30 kDa band (SDS–PAGE and PVDF blot) yielded the sequence Ile-Val-Gly-Gly-Glu-__-__-Pro-__-, consistent with the published sequence of mPR3 [22].

2.4. Purification of recombinant pro-enzymes expressed in 293 cells

Variants of hPR3, mPR3 and mNE carrying a carboxy-terminal cmyc-poly-His-tag were expressed in 293 cells. These are referred to as hPR3-cmyc, mPR3-cmyc, and mNE-cmyc, respectively. For purification of the pro-enzymes from stably transfected 293 cell clones, culture supernatants were dialyzed against 20 mM phosphate, 0.5 M NaCl, 10 mM imidazole, pH 7.4 (binding buffer), and applied to a HiTrap chelating HP column (Amersham Pharmacia Biotech Inc, NJ, USA). The columns were washed twice with binding buffer and once with 20 mM phosphate, 0.5 M NaCl, 50 mM imidazole, pH 7.4. Proteins were eluted in one step with 20 mM phosphate, 0.5 M NaCl, 500 mM imidazole (100 mM for mPR3-cmyc), pH 7.4.

2.5. Activation of purified pro-enzymes with cathepsin C

Because recombinant neutrophil serine proteases secreted by 293 cells are not amino-terminally processed, purified hPR3-cmyc, mPR3-cmyc and mNE-cmyc were activated in vitro by amino-terminal cleavage of the activation-dipeptide. For mPR3-cmyc and mNE-cmyc, 50 μg/ml of proenzyme were incubated with 1.5 U/ml of cathepsin C in 33 mM MES, 467 mM NaCl, 30 μM DTT, pH 4.5 for 20 h at RT. Under these conditions hPR3-cmyc does not activate very well. Hence, 50 μg/ml of pro-enzyme were incubated with 1.5 U/ml of cathepsin C in 33 mM NaOAc, 0.66 mM NaCl, 28 mM DTT, pH 5.5 for 8 h at RT. Cleavage of the activation dipeptide by cathepsin C was monitored by hydrolysis of MeOSuc-AAPV-pNA.

2.6. Determination of enzyme concentrations by active site titration

Each enzyme was labeled with >50x molar excess of ^{3}H-diisopropylfluorophosphate (^{3}H-DFP, Perkin/Elmer Life Sciences, Boston, MA). Enzymes were TCA-precipitated, radioactivity counted, and counts plotted against a standard curve of known trypsin concentrations, which were determined with cleavage of *p*-nitrophenyl *p*′-guanidinobenzoate [26]. Based on this active site titration, enzymes were diluted to the desired concentrations in 0.01% aqueous Triton X-100 and added to the assay buffers.

2.7. Enzyme activity assays

To characterize enzymatic activities of the different neutrophil serine proteases from both species, kinetic assays with the substrates MeO-Suc-AAPV-pNa, Suc-AAPV-pNa, MeOSuc-AIPM-pNa, Suc-AAPL-pNa, Suc-AAA-pNa, and Suc-AAPF-pNa (Bachem, King of Prussia, PA) were performed in 100 μl of 90 mM Tris, 52 mM NaCl buffer containing 0.5% Triton X-100 (w/v) and 10% dimethylformamide (v/v), pH 8.0. Except where indicated, values are expressed as the means(±standard error) of three separate determinations. The values of each determination represent the average of those estimated from linear regression analysis of Lineweaver–Burk, Hanes–Woolf and Eadie–Hofstee plots. V_{max} values were based on a molar absorbance of 2448 ($A_{405\,nm}$–$A_{490\,nm}$) for 100 μl of 4-nitroaniline in a microtiter plate well.

Bovine oxidized insulin B chain (30 μM, HPLC-purified) was digested with 3–25 nM enzyme in 0.08 M Tris, 0.1 M NaCl, pH 8. Reactions were terminated with glacial acetic acid. Digests were subjected to HPLC on a Vydac Protein and Peptide C_{18} column. Solvent A was 0.07% aqueous trifluoroacetic acid (TFA) and solvent B was 0.05% TFA in acetonitrile. Peptides were separated at a flow rate of 1 ml/min using a linear gradient from 10% B to 40 % B over 30 min (detection at 214 nm). Peaks were collected and peptides identified by amino acid analysis using acid hydrolysis.

Bovine fibrinogen (32 μM) was digested with 36 nM enzyme in 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.4 (TBS). At various intervals, the clotting time of the digested fibrinogen was measured in a CoaScreener Hemostasis Analyzer (American Labor Corp., Largo, FL); 50 μl of digest was added to 200 μl TBS, incubated for 2 min at 37 °C, and fibrin was generated by the addition of 5 μl of bovine thrombin. Similarly, aliquots of digests were taken at the specified times for SDS–PAGE (PhastSystem™, Amersham Biosciences, Piscataway, NJ). Enzymatic activity was stopped by dilution with reducing sample preparation buffer followed by heating for 10 min at 90 °C.

2.8. Inhibitor studies

Enzyme and inhibitor were incubated for 1 h at RT in 50 μl of 60 mM Tris, 80 mM NaCl, pH 8.0; residual enzymatic activity was determined by adding 50 μl of 2 mM MeOSuc-AAPV-pNA, 0.1 M

Tris, 1% Triton X-100 (w/v), 20% dimethylformamide (v/v), pH 8.0. Except where indicated, values are the mean (± standard error) of three determinations. Concentrations of α_1-antitrypsin and SLPI were determined by assay with trypsin, concentration of eglin C by assay with chymotrypsin, and concentrations of elafin and Val_{15}-aprotinin were based on suppliers' information. Concentrations of enzyme ranging from 3 to 93 nM were used.

3. Results and discussion

3.1. Effect of different processing of recombinant proteases on enzymatic activity

To obtain sufficient quantities of purified murine proteases for functional studies, two mammalian expression systems were employed [21,24]. Recombinant hPR3 and mPR3 were expressed in HMC-1 and 293 cells, and mNE-cmyc in 293 cells. In HMC-1 cells, a large proportion of the nascent serine protease is processed intracellularly to the active enzyme, which is stored in granules [21,22]. In contrast, recombinant neutrophil proteases expressed in 293 cells are mostly secreted into the cell supernatant as unprocessed pro-enzymes [24]. This allows modification of the unprocessed carboxy-terminus to facilitate subsequent purification [23], but in vitro cleavage of the amino-terminal pro-peptide is necessary for activation. The mature neutrophil serine proteases stored in HMC-1 cell granules and the pro-enzymes secreted into 293 cell supernatants also carry different Asn-linked glycans [21,24].

To ascertain potential effects of differences inherent to the expression systems, we first compared enzymatic activities of purified recombinant hPR3 and mPR3 expressed in the two different cell types. Digestion of oxidized insulin B chain by purified human PMN-PR3, the three different recombinant hPR3 variants, hPR3-salt, hPR3-Tx100 purified from HMC-1 cells, and hPR3-cmyc purified from 293 cells, revealed no significant qualitative or quantitative differences in protein-cleavage rates or pattern (Fig. 3A). Similarly, the protein-cleavage patterns and rates of mPR3 expressed in HMC-1 cells did not differ from those of mPR3-cmyc expressed in 293 cells (not shown).

The cellular origin of the proteases also did not significantly affect the kinetics of hydrolysis of MeOSuc-AAPV-pNa. K_{cat}/K_m values ($M^{-1}\,s^{-1}$) for the hPR3 variants were: 400 ± 22 (PMN-PR3, $n = 5$), 499 ± 27 (hPR3-salt, $n = 3$), 501 ± 24 (hPR3-T × 100, $n = 3$), and 283 ± 51, (hPR3-cmyc, $n = 3$). The K_{cat}/K_m values were 5464 ± 977 ($n = 3$) for mPR3 from HMC-1 cells, and 4193 ± 1116 ($n = 3$) for mPR3-cmyc from 293 cells, respectively. Similarly, the kinetics of hydrolysis of other substrates listed in Table 1 were equivalent for all four human PR3 variants (data not shown).

Our comparison of native purified human PMN-PR3 to the different recombinant hPR3 variants has several implications. First, it clearly indicates that neither the cleavage of the carboxy-terminal PR3 pro-peptide (occurring in PMN and HMC-1 cells), nor the addition of a carboxy-terminal peptide extension alter enzymatic activity significantly. Second, differences in Asn-linked glycans between rPR3 expressed in HMC-1 and 293 cells compared to PMN-hPR3 [21,24] have no apparent effect on hydrolytic activity. Finally, the different purification procedures have no effect. The comparison of recombinant mPR3 expressed in the two different expression systems further corroborates this. Because of these findings and because of the simplicity of purification of carboxy-terminally tagged protease variants expressed in 293 cells, we subsequently used recombinant mNE-cmyc for the direct comparison to native hNE purified from neutrophils (Fig. 4).

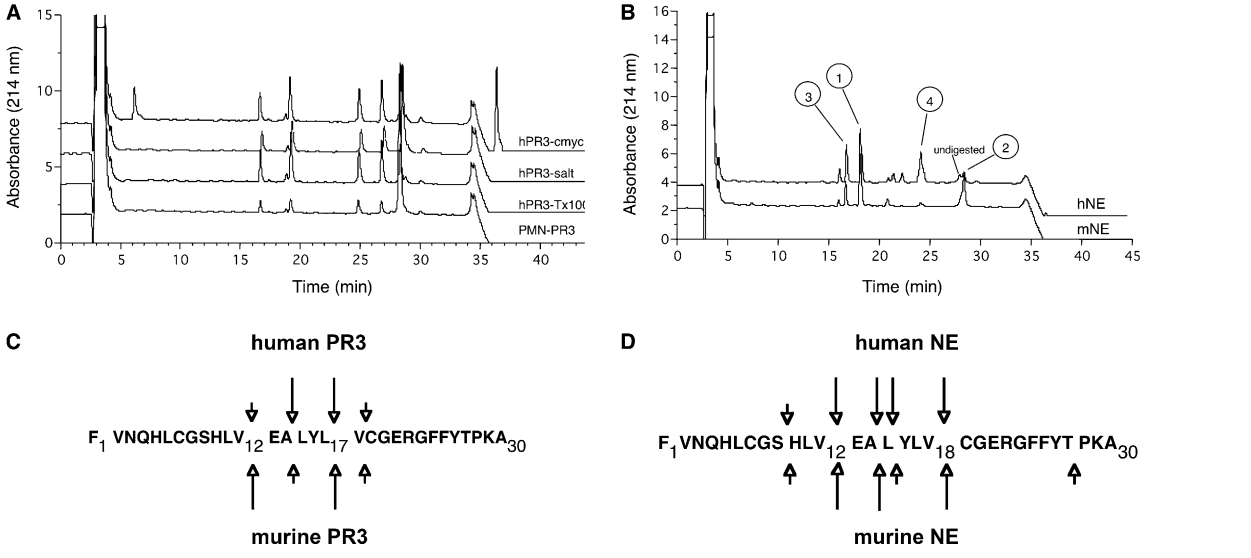

Fig. 3. Analysis of cleavage patterns and rates of oxidized insulin B chain. (A) HPLC elution profiles of oxidized insulin B chain peptides generated by different hPR3 variants. Elution profiles are from 8 h digests using 3.5 nM enzyme. All hPR3 variants generated the same peptides at the same cleavage rate. The early peak detected in the hPR3-cmyc chromatogram is not a peptide, but an unidentified component of the cathepsin C digest. (B) HPLC elution profiles of digests of oxidized insulin B chain peptides generated by hNE and mNE. These examples show 30 μM oxidized insulin B chain digested with 12 nM PMN-HNE (hNE) for 2 h, or with 25 nM mNE-cmyc (mNE) for 30 min. Four peptides are enumerated which define 2 of the early cleavage sites. Cleavage between Val_{18} and Cys_{19} produces peaks (1) Cys_{19}-Ala_{30} and (2) Phe_1-Val_{18}. Cleavage between Val_{12} and Glu_{13} produces peaks (3) Phe_1-Val_{12}, and (4) Glu_{13}-Ala_{30}. (C) Summary of HPLC analysis of time-course digests of insulin B-chain, 0.1–20 h, by hPR3 and mPR3. (D) Summary of HPLC analysis of time-course digests of insulin B-chain, 0.1–20 h, by hNE and mNE. Arrows indicate sites of rapid (long) and slow (short) cleavage.

Table 1
Kinetic parameters for murine and human PR3 and NE enzyme actvity

Substrate	Parameter[a]	Human PR3[b]	Murine PR3[c]	Murine NE[d]	Human NE[e]
MeOSuc-AAPV-pNA	K_{cat}	0.59 ± 0.04	6.2 ± 1.0	12.1 ± 2.5	9.2 ± 1.8
	K_m	1.2 ± 0.02	1.1 ± 0.1	0.52 ± 0.03	0.28 ± 0.02
	K_{cat}/K_m	499 ± 27	5464 ± 977	23273 ± 4538	33915 ± 5757
Suc-AAPV-pNA	K_{cat}	0.37 ± 0.08	11.2 ± 0.5	7.1 ± 0.9	5.7 ± 2.0
	K_m	3.7 ± 0.8	2.5 ± 0.3	0.59 ± 0.03	0.42 ± 0.08
	K_{cat}/K_m	98 ± 3	4692 ± 631	12251 ± 2076	13785 ± 3192
MeOSuc-AIPM-pNA	K_{cat}	0.26 ± 0.01	7.2 ± 1.9	1.7 ± 0.06	2.0 ± 0.6
	K_m	0.61 ± 0.12	1.5 ± 0.3	2.3 ± 0.3	1.2 ± 0.3
	K_{cat}/K_m	417 ± 20	4619 ± 175	767 ± 130	1693 ± 135
Suc-AAA-pNA	K_{cat}	Very low activity	0.031[f]	5.8 ± 1.0	1.2 ± 0.3
	K_m		0.61	5.9 ± 0.5	2.7 ± 0.2
	K_{cat}/K_m		51	966 ± 106	465 ± 103
Suc-AAPL-pNA	K_{cat}	Very low activity	0.16 ± 0.14[g]	0.67[f]	0.39[f]
	K_m		3.5 ± 2.6	5.5	3.5
	K_{cat}/K_m		53 ± 17	122	110
Suc-AAPF-pNA	K_{cat}	Not done	Not done	0.16[f]	Very low activity
	K_m			3.9	
	K_{cat}/K_m			41	

[a]Units for K_{cat}, K_m and K_{cat}/K_m are s^{-1}, mM, and $M^{-1}\ s^{-1}$, respectively.
[b]Data shown for human PR3 were obtained with rhPR3-salt purified from HMC-1/hPR3 cells.
[c]Data shown for murine PR3 were obtained with mPR3 purified from HMC-1/mPR3 cells.
[d]Data shown for or murine NE were obtained with mNE-cmyc purified from 293/mNE-cmyc cells.
[e]Data shown for human NE were obtained with purified PMN-HNE.
[f]One determination.
[g]Two determinations.

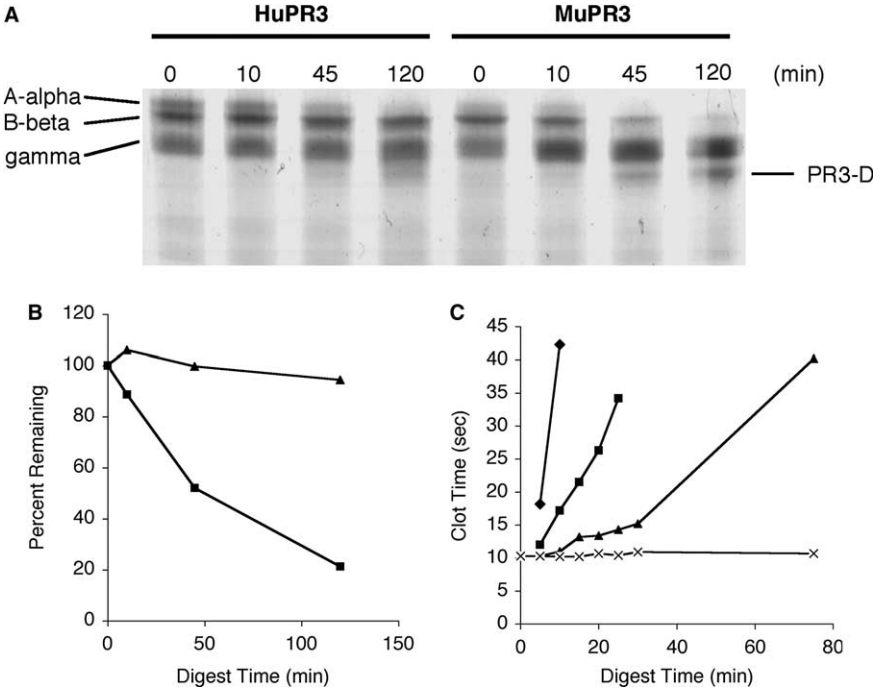

Fig. 4. Degradation of bovine fibrinogen. Bovine fibrinogen (32 μM) was digested with 36 nM enzyme. (A) 8–25% PhastGel™ (under reducing conditions) of bovine fibrinogen digested with PMN-hPR3 (lanes 1–4) and recombinant mPR3 (lanes 5–8). Samples were taken at 0 min (lanes 1 and 5), 10 min (lanes 2 and 6), 45 min (lanes 3 and 7) and 120 min (lanes 4 and 8). PR3-D, PR3 degradation product. (B) Relative disappearance of the B-beta-chain of bovine fibrinogen upon digestion with PMN-hPR3 (triangles) and recombinant mPR3 (squares) was quantified by image analysis of the scanned gel. (C) Thrombin-induced clotting times of undigested bovine fibrinogen (×) and of bovine fibrinogen digested with PMN-hNE (triangles), recombinant mPR3 (squares) and PMN-hPR3 (diamonds). Shown are representative examples of 3 (A and B) and 5 (C) repeat experiments.

3.2. Physico-chemical differences between hPR3 and mPR3

The purification of recombinant hPR3 and mPR3 from HMC-1 cells required different approaches. Purification of hPR3 was dependent on detergent in the cell lysis buffer. In contrast, mPR3 activity was recovered easily out of the HMC-1 cell supernatants after ethanol fixation of the cells. Unless the mPR3 expressing HMC-1 cells are exposed to this ethanol fixation procedure, the hydrolytic activity of mPR3 is lost within minutes. This suggests the presence of either an inhibitor or an inactivating protease for mPR3 in HMC-1 cells, which may gain access to the mPR3 as it is released much more easily from HMC-1 cell granules than its human homologue. Furthermore, the final purification steps for hPR3 consisting of cation-exchange chromatography and elution with a NaCl gradient (Fig. 2A) resulted only in incomplete elution of hPR3 between 0.5 and 1.5 M NaCl.

These findings indicate substantial differences in physico-chemical properties between hPR3 and mPR3. It appears that hPR3 is much more difficult to dislodge from its intracellular storage compartments in HMC-1 cells than mPR3. One possible explanation is the more basic nature of hPR3. The calculated isoelectric point (pI) for mPR3 is 6.7 compared to 7.7 for hPR3. Experimental evidence points towards an even more basic pI of 9.1 to >9.5 for hPR3 [27,28]. These findings suggest that differences between hPR3 and mPR3 with respect to granule release or interactions with cell surfaces can be expected under inflammatory conditions in vivo. Such differences may be one possible explanation for our observation that autoantibodies generated against mPR3 had significant pro-inflammatory effects in wild-type mice, but did not induce a vasculitic disease reminiscent of human ANCA-associated vasculitis [8].

3.3. Enzymatic activities of mPR3 and mNE compared to their human homologues

To characterize the enzymatic activities of mPR3 and mNE compared to their human homologues, we measured kinetics of hydrolysis of a variety of synthetic substrates (Table 1). MeOSuc-AAPV-pNA is cleaved 10 times more efficiently by mPR3 than by hPR3, and the efficiency of mNE and hNE for this substrate is even 4–6 times higher than that of mPR3, consistent with a larger S1 pocket of mPR3, mNE and hNE compared to hPR3. MeOSuc-AIPM-pNA is most efficiently cleaved by mPR3. Suc-AAA-pNA is a substrate for both mNE and hNE. Suc-AAPL-pNA is not a good substrate for any of the proteases; it is a poor substrate for mPR3 and not cleaved by hPR3. These data indicate that substrate specificity and efficiencies of substrate hydrolysis of mNE are very similar to hNE, thus supporting the validity of conclusions drawn from NE-deficient mice [12,14,15,29].

In contrast, mPR3 differs more substantially from hPR3. As mPR3 seems to display some more "elastase-like" properties, the distinction of mPR3 effects from mNE effects in murine models seems important. Our finding that MeOSuc-AIPM-pNA is a much better substrate for mPR3 than for mNE may help in this respect. However, more clearly discriminating substrates such as recently developed for hPR3 and hNE [30] remain to be identified or developed.

Oxidized insulin B chain is characterized as a large peptide substrate for elastases and PR3 [2,31]. Representative chromatograms of digests of oxidized insulin B chain are shown in Fig. 3. Using similar substrate and enzyme concentrations throughout, chromatograms were compared at time points ranging from 6 min to 20 h. Peptides were identified by amino acid analysis and quantified by peak height. Fig. 3C and D summarize these HPLC analyses over time for human and murine PR3 and NE.

Both hPR3 and mPR3 cleaved rapidly at Leu_{17}-Val_{18}, and the resulting carboxy-terminal peptide was slowly digested at Val_{18}-Cys_{19}, yielding a stable peptide Cys_{19}-Ala_{30}. The amino-terminal peptide (Phe_1-Leu_{17}) was cleaved by both enzymes at Ala_{14}-Leu_{15} (much more rapidly by hPR3) and at Val_{12}-Glu_{13} (somewhat more quickly by mPR3).

Both hNE and mNE cleaved insulin B chain very efficiently at Val_{18}-Cys_{19}, resulting in a stable Cys_{19}-Ala_{30} peptide (which was very slowly cleaved at Thr_{27}-Pro_{28} by mNE, but not by hNE) and the Phe_1-Val_{18} peptide. This amino-terminal peptide was quickly digested further at Leu_{15}-Tyr_{16} (more slowly by mNE), Ala_{14}-Leu_{15}, and Val_{12}-Glu_{13}. Both mNE and hNE cleaved very slowly at Ser_9-His_{10}.

The insulin B chain cleavage results indicate that all four isozymes have unique enzyme characteristics that are more complex than predictable on the basis of S1 selectivity. Other factors may contribute substantially to the preferred binding of substrates (or inhibitors) to the enzymes. These may include the unpredictable role of van der Waals interactions between substrate and enzyme [32], or interactions between substrate and differing structures on the prime side of the substrate pocket [30,33]. Consequently, screens of the substrate space using positional scanning-synthetic combinatorial libraries appear necessary to identify optimal specific substrates (and inhibitors) for each of the four homologue neutrophil serine proteases [34].

In vitro experiments have identified a variety of matrix proteins, cytokines and clotting factors as potential substrates for human NE or PR3. Yet, little is known about which of these molecular substrates are really physiologically relevant substrates for PR3 and NE in vivo. Furthermore, they may be different in different species. Therefore, we chose bovine fibrinogen as a potentially "physiologic" large molecular substrate for additional comparative studies of enzymatic activity. First, fibrinogen has been described as a relevant substrate for NE, and second, fibrinogen is highly conserved across species. Again, as shown in Fig. 4, mPR3 behaves differently from hPR3, and more like hNE.

3.4. Inhibition of mPR3 and mNE compared to their human homologues

To identify common and discriminating inhibitors, we determined the molar equivalents of inhibitors required to reduce the enzymatic activity (IC_{50}-values) of the four proteases (Table 2). Inhibitor studies comparing the IC_{50}-values of the different hPR3 and mPR3 variants (purified native PMN-PR3, cathepsin C-activated recombinant hPR3-cmyc and mPR3-cmyc from 293 cells) showed the same species-specific relationships as the ones shown in Table 2. As expected, α_1-antitrypsin inhibited all of the serine protease homologues well. Perhaps in part because of its small P1 residue (Ala), elafin also inhibited all of the enzymes well.

As reported [35], SLPI inhibited hNE and mNE well. It did not inhibit hPR3 and mPR3. This observation indicates that SLPI may be a useful tool to distinguish between mNE and mPR3 activity in biological specimens obtained from mice.

Table 2
Molar equivalents of inhibitor required to reduce the enzymatic activity of human and murine PR3 and NE by fifty percent

Inhibitor	Human PR3[b]	Murine PR3[c]	Murine NE[d]	Human NE[e]
Val_{15}-aprotinin	LI[a]	447 ± 153	142 ± 101	0.81 ± 0.37
SLPI	LI[a]	LI[a]	0.61 ± 0.3[f]	0.60 ± 0.07[f]
Eglin C	117 ± 72	3.3 ± 1.1	6.0 ± 3.0	0.57 ± 0.13
α-1 Antitrypsin	0.98 ± 0.29	0.74 ± 0.17	0.43 ± 0.03	1.1 ± 0.2
Elafin	1.9 ± 0.1[f]	0.99 ± 0.02	1.0 ± 0.3[f]	1.4 ± 0.3

[a]Limited inhibition; >75% of activity remained in the presence of the highest concentration of inhibitor.
[b]Data shown for human PR3 were obtained with rhPR3-salt purified from HMC-1/hPR3 cells.
[c]Data shown for murine PR3 were obtained with rmPR3 purified from HMC-1/mPR3 cells.
[d]Data shown for or murine NE were obtained with rmNE-cmyc purified from 293/mNE-cmyc cells.
[e]Data shown for human NE were obtained with purified PMN-HNE.
[f]Two determinations.

Eglin C, which like SLPI has a Leu at P1, was a potent inhibitor of hNE, a significant inhibitor of mNE and mPR3, a poor inhibitor of hPR3. An even more striking species difference between hNE and mNE was observed with the custom-designed hNE inhibitor, Val_{15}-aprotinin. This observation further supports the notion that factors beyond P1–S1 interactions substantially influence the interactions between enzymes and substrates as well as inhibitors [30,32,33]. Consequently, custom-designed inhibitors of human neutrophil serine proteases need to be evaluated against their species-specific homologues before conclusions about biological or therapeutic effects of such inhibitors can be extrapolated from murine models. Our recombinant expression systems serve as a convenient source for purified murine neutrophil serine proteases required for such experiments.

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