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Published in:
Clinical and Experimental Immunology

DOI:
10.1046/j.1365-2249.2003.02083.x

2003

Clin Exp Immunol 2003; **131**:528–535

Increased circulating levels of proteinase 3 in patients with anti-neutrophilic cytoplasmic autoantibodies-associated systemic vasculitis in remission

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(Accepted for publication 28 November 2002)

SUMMARY

In systemic small vessel vasculitides, patients form autoantibodies against neutrophil granular proteins, anti-neutrophilic cytoplasmic autoantibodies (ANCA). Some correlation is seen between ANCA titre and disease activity, but whether this is cause or effect is still unknown. It has been reported that levels of proteinase 3 (PR3), one of the main ANCA antigens, are increased in patients with active disease. An increased level of circulating antigen could mean a predisposition to autoimmunity. In order to explore this we measured PR3 levels in patients with stable disease. In addition we measured neutrophil gelatinase-associated lipocalin (NGAL) as a specific marker of neutrophil degranulation, cystatin C as a marker of renal function as well as C-reactive protein (CRP), IL-6 and sTNFr1 as markers of inflammation. PR3, NGAL, IL-6 and sTNFr1 were measured in plasma by the ELISA technique. In the PR3 ELISA, we used anti-PR3 monoclonal antibodies as capture-antibodies and affinity-purified rabbit-anti-PR3 antibodies for detection. PR3-ANCA, myeloperoxidase (MPO)-ANCA, CRP and cystatin C were measured by routine methods. PR3 was significantly raised ($P < 0{\cdot}0001$) in vasculitis patients (median 560 μg/l, range 110–3940, $n = 59$) compared with healthy blood donors (350 μg/l, 110–580, $n = 30$) as well as disease controls (360, 110–580, $n = 46$). No correlation was seen with disease activity, inflammation or renal function. The raised NGAL levels correlated strongly with decreased renal function ($r = 0{\cdot}8$, $P < 0{\cdot}001$). After correcting for this, slightly increased levels (110, 42–340, $n = 59$) were observed compared with healthy blood donors (81, 38–130, $n = 25$), but not compared with the disease controls (120, 57–260, $n = 48$). In the disease controls, there was a significant correlation between NGAL and proteinase 3 ($r = 0{\cdot}3$, $p < 0{\cdot}05$), but this was not the case in the vasculitis patients. Whether patients had PR3-ANCA or MPO-ANCA was of no significance. In our measurements, we found significantly raised levels of PR3 in plasma from patients with small vessel vasculitis, regardless of ANCA specificity. This was not due to decreased renal function, ongoing inflammation or neutrophil activation. Plausible mechanisms for this include defects in the reticuloendothelial system, genetic factors and selective neutrophil degranulation or leakage.

Keywords autoimmunity ANCA neutrophil proteinase 3 systemic vasculitis

INTRODUCTION

The most common forms of small vessel vasculitis are microscopic polyangitis (MPA) and Wegener's granulomatosis (WG). Patients with these diseases make autoantibodies against neutrophil granular proteins, so-called ANCA (anti-neutrophilic cytoplasmic autoantibodies), the main autoantigens being myeloperoxidase (MPO) and proteinase 3 (PR3). Some correlation is seen between autoantibody production and clinical course, but the aetiology is still unknown [1]. PR3 is a 29-kDa serine protease, stored mainly in neutrophil azurophil granules, but also present in the secretory and specific granules as well as in the granules of monocytes [2]. It is bound in the circulation to alpha1-antitrypsin, but is also present on the surface of primed or apoptotic human neutrophils in a bioactive form [3,4]. Alpha-1-antitrypsin can be inactivated by reactive oxygen species, produced by, for example, MPO. This would lead to an acquired localized protease/antiprotease imbalance which could be further enhanced by proteinase 3/PR3–ANCA interactions [5,6]. The deficient PI-Z alpha-1-antitrypsin phenotype is associated with increased frequency as well as a poorer prognosis for ANCA-positive systemic vasculitis [7]. Besides its proteolytic effects, PR3 exhibits chemotactic properties and has an influence on the proliferation of granulopoietic progenitor cells [2]. The gene is localized on chromosome 19p13.3 and it has been demonstrated recently that a specific polymorphism in the PR3 promoter is associated with WG [8]. This

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polymorphism leads to a new transcription factor binding site in the promotor and could generate PR3 overexpression. Moreover, PR3 is involved in cytokine and chemokine activity regulation and the processing of cytokine binding proteins, indicating a regulatory role in inflammatory processes [9].

The neutrophil has different granules, the main subsets of which have distinct functional differences and are mobilized at different times during activation. The contents of the azurophil granules are the last to be mobilized and take part primarily in destruction and digestion in the phagolysosome [10]. Neutrophil gelatinase-associated lipocalin (NGAL), localized in the more easily mobilized specific granules, is an acute phase protein, released from neutrophils upon stimulation by phagocytosis. It has a half-life of 10 min in the circulation, reflecting redistribution to the extravascular compartments as well as renal elimination *per se* [11,12]. This lipocalin is today well established as a marker of neutrophil activation and degranulation [13]. Tumour necrosis factor (TNF) is a potent inflammatory cytokine produced by various cells, mainly activated mononuclear leucocytes [14]. Soluble TNF receptor-1 p55 (sTNFR-1), that sheds extracellular portions of the TNF receptor, is secreted mainly by mononuclear cells. Its concentration has been found to correlate well with that of TNF, thus probably reflecting the activation status of the TNF/TNF-receptor system, and hence mononuclear cell activation [15]. IL-6 is a pleiotropic cytokine and an integral mediator of the acute phase response [16].

It has been reported that patients with systemic small vessel vasculitis have raised levels of PR3, a finding that might signify a predisposition to autoimmunity [17,18]. These studies were, however, performed on patients with active disease and a runaway inflammatory cascade. In order to approach the pathophysiology of ANCA-associated vasculitis, the aim of this study was to investigate further the possibility that patients in a stable phase have increased levels of circulating PR3. We have also tried to address possible explanations for this, such as neutrophil activation, inflammatory activity and decreased renal filtration.

MATERIALS AND METHODS

Patient material

Fifty-nine patients with ANCA-associated systemic vasculitis, none of whom were on dialysis or suffering from any bacterial or viral infections, were included in this study. All were well known at the clinic and in a stable phase at the time of sampling (1995–96). Based on clinical observations performed by their usual doctors at the Department of Nephrology, Lund University Hospital, their initial status was classified as either remission (BVAS 0–1) or smouldering (BVAS 2–5) activity. The patients were followed-up after 6 years. At that time, the number of relapses as well as the development of any severe organ damage (cerebrovascular catastrophe, acute myocardial ischemia, renal failure), initiation of dialysis or death due to vasculitic complications were recorded. Relapse was defined as reappearance of symptoms and clinical findings in line with vasculitic disease, leading to a major change in immunosuppressive medication. These studies were performed without access to the PR3 results. The patients were grouped according to ANCA specificity (PR3 or MPO). Our two control groups consisted of 30 healthy blood donors (HBDs) and 48 disease controls (DCs). The latter were patients with kidney transplants who had undergone transplantation for reasons other than vasculitis or glomerulonephritis (14 had polycystic disease, 15 had diabetes, three had nodular sclerosis and 16 had other diagnoses). The DCs were selected with regard to similarities with our vasculitis patients, in terms of immunosuppressive medication and decreased renal function (Table 1).

The Helsinki Declaration regarding the use of human plasma was strictly observed.

Blood samples

Venous blood from all subjects was obtained in EDTA tubes. The blood was kept at 4°C and centrifuged within 3 h. The plasma was carefully aspirated with a Pasteur pipette and stored at –20°C until assayed. Plasma was used because PR3 is released from neutrophils during coagulation and serum can therefore not be used.

Purification of rabbit anti-PR3 antibodies

Construction of a PR3 column. One ml (1 mg/ml) PR3 (Wieslab AB, Lund, Sweden) was diluted in 1 ml 50 mM Tris-HCl (pH 8·0, 0·5% NaN_3, 1% Triton X100) and dialysed against PBS containing 1 mM PMSF in order to block the active site of PR3. Two ml Mini Leak gel (KemEnTech) was put into a 15-ml tube. After washing the gel (2×1 ml distilled H_2O, centrifugation 300 r.p.w. for 5 min), the PR3 dialysate was added to the Mini Leak

Table 1. Patients and controls

	Remission	Smouldering	DC	HBD
Total no.	44	15	48	30
No. MPO/PR3/MPO + PR3 (*n*)	15/28/1	8/7/0	–	–
No. females/males (*n*)	21/23	5/10	17/31	–
Age (years)	57 (22–84)	63 (27–82)	51 (21–81)	–
Relapses in 6 years (*n*)	18	8	–	–
Severe outcome (*n*)	9	15	–	–
Cystatin C (mg/ml)	1·5 (0·4–4·6)	1·9 (0·8–5·1)	1·5 (0·7–5·3)	0·7 (0·4–1·2)
BVAS	0–1	2–5	–	–

HBD = healthy blood donors, DC = disease controls. MPO = anti myeloperoxidase-ANCA positive patients, PR3 = anti proteinase 3-ANCA positive patients, MPO + PR3 = double positive patients. Severe outcome = severe organ damage, start in dialysis or death due to vasculitic complications during 6 years follow-up. BVAS = Birmingham vasculitis activity score.

gel together with 0·85 ml coupling buffer [3·5 M (NH_4) $2SO_4$ pH 8·8]. The sample was then left to incubate overnight on a rocking table at RT. The gel was then put into a glass column (1 cm in diameter) and rinsed with coupling buffer for 1 h, flow 20 ml/h, followed by 0·1 M ethanolamine-HCl, the latter in order to block active groups on the gel. Finally, the gel was equilibrated in 50 mM Tris-HCl pH 7·0, 0·15 M NaCl, 0·02% NaN_3.

Affinity purification of rabbit anti-PR3 serum. The purification of rabbit anti-PR3 antibodies was performed in a biological HR system from Bio-Rad, Solna, Sweden.

Six ml rabbit anti-PR3 serum (Wieslab AB) was dialysed against phosphate buffer (20 mM Na-phosphate pH 7, 0·01% NaN3) before loading onto a protein G column (6 ml Protein G sepharose from Pharmacia). Rinsing with phosphate buffer was then followed by elution with 0·1 M Glycin-HCl, pH 2·7. The protein G elution was dialysed against 50 mM Tris-HCl (pH 7·0, 0·15 M NaCl, 0·02% NaN3). The absorbance was measured at 280 nm and the amount of immunoglobulins obtained was calculated to be 24 mg. The protein G elution was then affinity-purified on the Mini Leak PR3 column (see above). Two mg IgG was loaded in every run, rinsed with 50 mM Tris –HCl, and eluted with 6 M urea, 50 mM citrate, pH 4·0. The elutions were pooled, dialysed against PBS and concentrated with in a Vivaspin centrifugal concentrator (Vivascience, Hanover, Germany) to 0·2 mg/ml in a volume of 6·5 ml.

ELISA assays

PR3. A microtitre plate (Nunc immunoplate) was coated overnight with 100 µl/well of a purified mixture of two monoclonal PR3 antibodies, 4A3 and 4A5 [19] – each 1·5 µg/ml in coating buffer (0·01 M Na2CO3, 0·04 M NaHCO3, 0·02% NaN3, pH 9·5–9·7) at 4°C. The monoclonal antibodies recognize the mature as well as the proform of PR3 [20,21]. The plate was blocked with sample buffer (PBS 7·3–7·4, 0·05% Tween 20, 0·2% BSA) for 1 h and washed with 0·9% NaCl, 0·05% Tween 20 three times. All subsequent incubations were performed in 100 µl volumes at RT on a rocking table and followed by washing three times. Plasma samples diluted to 1/20, 1/40 and 1/80 in sample buffer were added and the plate incubated for 1 h. After washing, bound PR3 was detected by 1 h incubation with affinity-purified rabbit anti-PR3 diluted to 1/600 in sample buffer. Washing was followed by the addition of the conjugate (alkaline phosphatase-labelled swine antirabbit IgG from Sigma-Aldrich, Stockholm, Sweden), diluted to 1/1000 in sample buffer and 1 h incubation. P-nitrophenyl-phosphate disodium (Sigma) 1 mg/ml in substrate buffer (1·0 M diethanolamine, pH 9·8, 0·5 mM $MgCl_2$, 0·02% NaN3) was used as substrate and optical densities were read at 405 nm. A standard curve was produced by incubation of a two-fold dilution series of PR3 (Wieslab AB), starting with 1 µl PR3 (100 µg/ml) in 499 µl sample buffer (0·2 µg/ml). Recovery experiments were performed by adding 10 and 100 ng/ml PR3 to a plasma sample and, after incubation at RT for 30 min, performing the assay as described.

Detection of PR3/alpha1-antitrypsin complexes was also performed using an ELISA with the design described, except that the detecting antibody was rabbit antialpha1-antitrypsin (Dako, Glostrup, Denmark) instead of rabbit anti-PR3, diluted to 1/8000 in sample buffer [17].

NGAL, sTNFr1. NGAL and sTNFr1 were measured with ELISAs using monoclonal or polyclonal antibodies as catching antibodies, as described earlier [12,22].

Il-6. A quantitative sandwich enzyme immunoassay, in which a monoclonal antibody specific to IL-6 had been precoated on a microplate, was obtained commercially from R&D systems (Abingdon, UK) and used according to the manufacturer's specifications.

Cystatin C and C-reactive protein. The Clinical Chemical Laboratory at Lund University Hospital, Lund, Sweden, performed analyses on a Hitachi 917 Pluto. Kits from Roche Diagnostics and Dako were used.

ANCA. Wieslab AB, Lund, Sweden, performed analyses of PR3-ANCA, MPO-ANCA and capture PR3-ANCA by routine methods [23,24].

Statistical analysis

All statistical analyses were performed in StatView 5·01. The correlation analyses, the simple regression analyses and the correlation *Z*-tests were performed on logarithmic data. One-way analysis of variance was performed using ANOVA with Fisher's PLSD on logarithmic data.

RESULTS

PR3 levels in plasma

In our measurements, we found that PR3 was significantly raised ($P < 0·0001$) in vasculitis patients (median 560 µg/l, range 110–3940, $n = 59$) compared with healthy blood donors (350, 110–580, $n = 30$) as well as disease controls (360, 110–580, $n = 46$) (Fig. 1, Table 2a). The results of the alpha1 antitrypsin/PR3-complex ELISA correlated well, as expected, with those of the PR3 ELISA ($r = 0·8$, $P < 0·0001$). The vasculitis patients had a tendency to divide into two groups, one with normal proteinase 3 levels (450, 110–600, $n = 34$) and the other with raised levels (920, 610–3940, $n = 27$). These two groups were compared to each

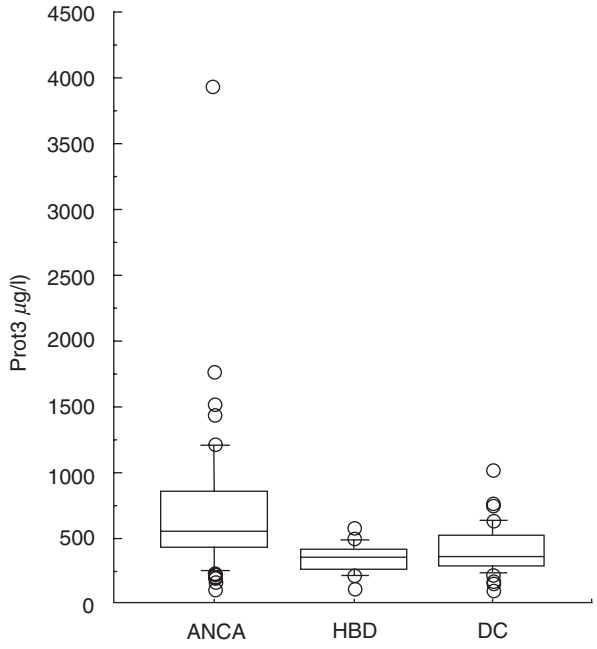

Fig. 1. Boxplot showing increased proteinase 3 levels in the patients with ANCA-associated systemic vasculitis (ANCA), compared to healthy blood donors (HBD) and disease controls (DC).

Table 2a. Plasma PR3 and ANCA levels

	Vasc-pat, total	MPO-pos	PR3-pos	DC	HBD
Prot 3 (μg/l),median (min–max)	560 (110–3940)	560 (380–1770)	570 (110–3940)	360 (110–1020)	350 (110–580)
MPO-ANCA (U)	–	79 (0–1400)	–	–	–
PR3-ANCA (U)	–	–	18 (0–77)	–	–

Vasc-pat total = all vasculitis patients, MPO-pos = MPO-ANCA positive patients, PR3-pos = PR3-ANCA positive patients, DC = disease controls, HBD = healthy blood donors. U = units.

Table 2b. Plasma PR3 and ANCA levels, Spearman's rank correlation

Non-parametric analysis	Rho	*P*-value
Proteinase 3, MPO-ANCA	0·4	0·04 (s.)
Proteinase 3, PR3-ANCA	– 0·4	0·04 (s.)

Rho = correlation coefficient, s. = significant.

other, when elucidating possible associations with high circulating levels of PR3.

PR3 versus *ANCA and renal function*

We found it interesting to look at a possible correlation between raised PR3 levels and ANCA titre. Whether patients had PR3- or MPO-ANCA was of no obvious importance. The two groups had similar PR3 levels. A positive correlation was seen with MPO-ANCA ($r = 0{\cdot}4$, $P < 0{,}05$) while there was, however, a negative correlation with PR3-ANCA ($r = -0{\cdot}4$, $P < 0{\cdot}05$) (Table 2b).

We also wanted to investigate whether the PR3 levels were influenced by renal function or not. No correlation was seen between poor renal function and plasma levels of PR3, using cystatin C as marker of renal filtration.

PR3 versus *inflammatory influence and mononuclear cell activation*

Raised PR3 levels could be part of general ongoing inflammatory activity. However, we could not find any correlation between the PR3 levels and IL-6 or CRP (Table 3a,b). No differences were found in IL-6 and CRP levels between patients with normal and patients with high PR3 levels.

Another possibility could be PR3 release from activated monocytes. STNFr1 was measured as a marker of monocytic activation. The sTNFr1-levels were raised in the vasculitis patients, but after correction for decreased renal filtration (the sTNFr1 values were divided by the cystatin *C*-values, giving us the parameter sTNFr1/C) there was no increase compared with healthy blood donors and disease controls (Fig. 2, Table 4a-b).

PR3 versus *neutrophil activation and degranulation*

Degranulating activated neutrophils are also a possible PR3 source and NGAL is a marker of neutrophil activation and degranulation. Greatly elevated NGAL levels were seen in our patients. There was, however, a strong positive correlation with decreased renal function ($r = 0{\cdot}8$, $P < 0{\cdot}001$) (Fig. 3). After

Table 3a. Biochemical markers of inflammation in plasma

	IL-6 (ng/l) Median (range)	CRP (mg/l) Median (range)
Vasc pat ($n = 59$)	3·6 (0·8–25·5)	8·5 (0·0–35·4)
Smouldering ($n = 15$)	7·0 (1·9–25·5)	16·2 (3·2–35·4)
Remission ($n = 44$)	2·4 (0·8–7·7)	6·0 (0·0–29·1)
HighProt3 ($n = 25$)	3·6 (0·8–25·5)	9·7 (0·0–33·6)
NormProt3 ($n = 34$)	3·6 (1·0–16·6)	7·6 (0·0–35·4)
DC ($n = 48$)	3.9 (1·3–14·6)	–
HBD ($n = 30$)	1·0 (0·3–2·4)	1·6 (0·0–8·0)

Vasc pat = all vasculitis patients. Smouldering: patients with smouldering disease activity. Remission = patients in remission. HighProt3 = patients with proteinase 3 levels >2 s.d. above the HBD mean value. NormProt 3 = patients with proteinase 3 levels ± 2 s.d. of the HBD mean value. DC = disease controls. HBD = healthy blood donors.

Table 3b. Correlation between plasma PR3 and markers of inflammation

	r	*P*-value
PR3 vs. IL-6	– 0·04	0·77 (n.s.)
PR3 vs. CRP	0·14	0·33 (n.s.)

r = correlation coefficient, n.s. = non-significant.

correction for this (the PR3 values were divided by the cystatin *C*-values, giving us the parameter NGAL/C) slightly increased levels (110, 42–340), $n = 59$) were still seen compared with healthy blood donors (81, 38–130, $n = 25$), but not compared with the disease controls (120, 57–260, $n = 48$) (Fig. 4). NGAL/C and PR3 showed a positive correlation in the disease control group ($r = 0{\cdot}3$; $P < 0{\cdot}05$), but not among the vasculitis patients (Table 4a,b). No differences were found between patients with normal and patients with high PR3 levels.

Clinical aspects

In comparison with clinical data, there was no significant difference in PR3 levels between patients in remission and patients with grumbling disease, nor did the PR3 levels predict who would develop vasculitic complications and/or have relapses (Table 5). The patients were followed-up after 6 years. At that time, 15 of 15

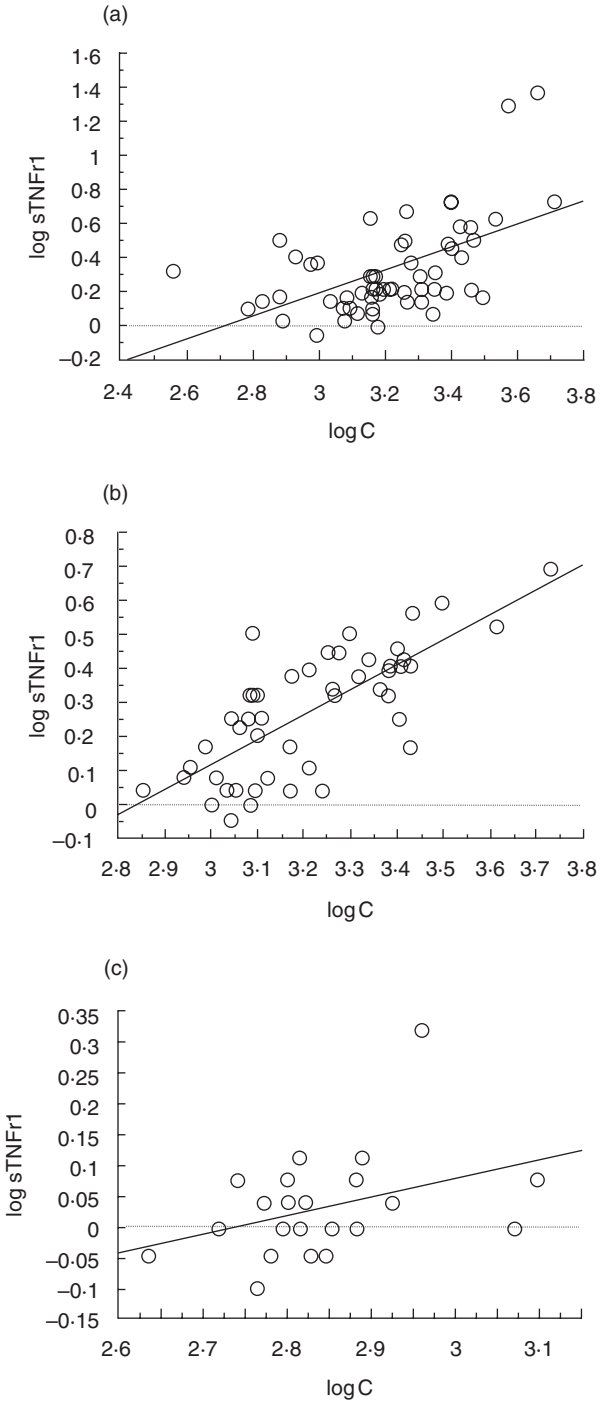

Fig. 2. Influence of renal filtration on sTNFr1 levels in plasma. The influence is strong in the patient groups, but less convincing in the normal control group. (a) Patients with systemic small vessel vasculitis, $r = 0{\cdot}54$, $P < 0{\cdot}0001$. (b) Disease controls, $r = 0{\cdot}74$, $P < 0{\cdot}0001$. (c) Healthy blood donors, $r = 0{\cdot}37$, $P = 0{\cdot}074$.

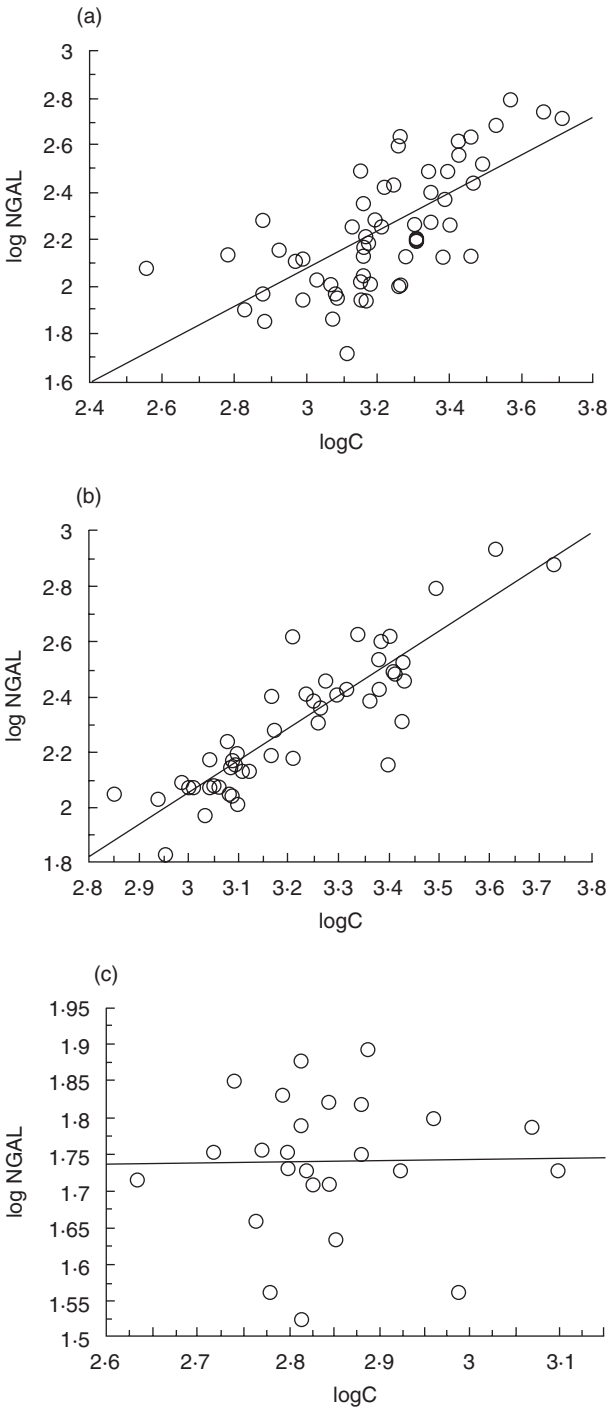

Fig. 3. Influence of renal filtration on NGAL levels in plasma. The impact is considerable in the patient groups, but absent in the normal control group. (a) Patients with systemic small vessel vasculitis, $r = 0{\cdot}69$ $P < 0{\cdot}0001$. (b) Disease controls, $r = 0{\cdot}88$, $P < 0{\cdot}0001$. (c) Healthy blood donors, $r = 0{\cdot}014$, $P = 0{\cdot}95$.

patients with smouldering disease activity at the time of sampling either had developed severe organ damage (cerebrovascular catastrophe, acute myocardial ischemia, renal failure) or had started in dialysis or had died due to vasculitic complications (Table 1).

DISCUSSION

The focus of this paper is on systemic small vessel vasculitis – a group of inflammatory, relapsing-remitting disorders of unknown aetiology, associated with autoantibody production. The elucidation of possible pathogenic mechanisms might help identify future

Table 4a. Neutrophilic and mononuclear cell markers of activation

	NGAL (μg/l) Median (range)	NGAL/C Median (range)	STNFr1 (μg/l) Median (range)	STNFr1/C Median (range)
Vasc-pat	160 (54–620)	110 (42–340)	2·9 (0·9–23·9)	1·6 (0·5–6·1)
DC	180 (66–870)	120 (57–260)	2·1 (0·9–5·0)	1·2 (0·6–2·6)
HBD	54 (34–176)	81 (38–130)	1·1 (0·8–2·1)	1·6 (0·8–2·3)

Vasc-pat = all vasculitis patients. DC = disease controls, HBD = healthy blood donors. NGAL/C = NGAL divided by cystatin C. sTNFr1/C = sTNFr1 divided by cystatin C.

Table 4b. Correlation between plasma PR3 and markers of leucocyte activation

	Vasc-pat CC (95% CI)	DC CC (95% CI)	HBD CC (95% CI)
PR3 *versus* NGAL	0·04 (– 0·22–0·29) n.s.	0·26 (– 0·03–0·51) n.s.	0·10 (– 0·28–0·46) n.s.
PR3 *versus* NGAL/C	0·11 (– 0·15–0·36) n.s.	0·30 (0·01–0·55) s.	–0·03 (– 0·42–0·37) n.s.
PR3 *versus* sTNFr1	–0·07 (– 0·32–0·19) n.s.	0·08 (– 0·22–0·36) n.s.	0·15 (– 0·24–0·50) n.s.
PR3 *versus* sTNFr1/C	–0·04 (– 0·29–0·23) n.s.	–0·10 (– 0·38–0·19) n.s.	–0·12 (– 0·5–0·30) n.s.

Vasc-pat = all vasculitis patients. DC = disease controls. HBD = healthy blood donors. CC = correlation coefficient. 95% CI = 95% confidence interval. NGAL/C = NGAL divided by cystatin C. sTNFr1/C = sTNFr1 divided by cystatin C. n.s. = non-significant, s. = significant.

Table 5. Comparison of PR3 levels in different clinical subgroups

	Fisher's PLSD, *P*-value
Vasc-pat *versus* HBD	<0·0001 (s.)
Vasc-pat *versus* DC	<0·0001 (s.)
Vasc-pat, remission *versus* smouldering	0·21 (n.s.)
Vasc-pat, severe outcome *versus* non-severe outcome	0·44 (n.s.)
Vasc-pat, relapse *versus* no relapse	0·74 (n.s.)

Vasc-pat = all vasculitis patients. HBD = healthy blood donors. DC = disease controls. Severe outcome = severe organ damage, start in dialysis or death due to vasculitic complications during 6 years' follow-up. Relapse = at least one relapse during 6 years' follow up. n.s. = non-significant, s. = significant.

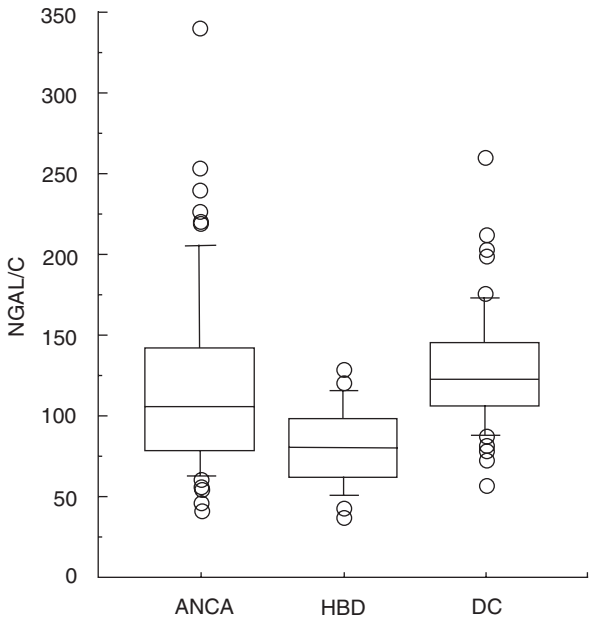

Fig. 4. Boxplot showing NGAL divided by cystatin C, in order to correct for decreased renal filtration. NGAL/C is moderately increased in patients with ANCA-associated systemic vasculitis (ANCA) and disease controls (DC), compared to healthy blood donors (HBD).

means of therapy and diagnosis. One of the main autoantigens, PR3, was found to be significantly raised in vasculitis patients compared with healthy blood donors and disease controls. The PR3-ELISA used is well established and comprises highly specific antibodies [17]. Little has been published on this subject. Haubitz *et al*. have conducted some analogue work on elastase, demonstrating elevated plasma levels in patients with systemic vasculitis [25]. Elevated PR3 levels in patients with systemic vasculitis were demonstrated in 1994 by Baslund *et al*. (153–1050 μg/l) [17] and Henshaw *et al*. (70–460 mg/l) ([18]. However, the latter measurements were performed on serum and not plasma. Both studies comprised rather small patient groups (n = 9–12) and focused on patients with active disease. In contrast, we have studied 59 patients in a stable phase – either remission or smouldering activity. In order to avoid the immunosuppressive medication bias, we chose a disease control (DC) group with a therapy similar to our patients'. The DC also had decreased renal filtration to a similar extent. Earlier work have shown raised PR3 levels in

patients with acute pancreatitis (254 ± 33 μg/l) as well as in infectious diseases (91–1150 μg/l [17,26].

Raised PR3 levels in vasculitis patients could have several explanations, such as increased production or decreased elimination, inflammatory activity and neutrophil or mononuclear cell leakage/degranulation. In our study, the high PR3 levels could not be explained by decreased renal function since there was no correlation at all with cystatin C. Whether patients had PR3- or MPO-ANCA was of no obvious importance. The two groups had similar PR3 levels. This, of course, makes further studies of MPO levels very interesting. MPO-ANCA showed a positive correlation with PR3, whereas there was a negative correlation between PR3-ANCA and PR3. This probably reflects that PR3 in complex with ANCA was not seen clearly in our ELISAs. No clear correlation was seen with the degree of inflammation (CRP, IL-6) or the disease activity, using BVAS. Elevated NGAL and sTNFr1 levels correlated strongly with decreased renal function. After correction, only moderately increased values were seen for NGAL/C compared with HBD and not at all compared with DC. In the DC group, but not in the vasculitis patients, NGAL/C and PR3 showed a significant positive correlation. This would indicate that the slightly elevated PR3 levels in the DC group could be explained by, for example, a therapy-related neutrophil influence, whereas there must be another explanation for this in the vasculitis patients. This could involve decreased elimination due to disease-related PR3/PR3-alpha1-AT complex receptor defects or changes in the reticuloendothelial system, or up-regulated production [8,27]. Theoretically, of course, selective azurophil degranulation or some sort of PR3 leakage might be conceivable reasons for this. Circulating neutrophils from patients have been demonstrated to express an increased level of PR3 and a high percentage of PR3-expressing neutrophils is a risk factor for vasculitis [28]. However, according to our results, this would not be connected with neutrophil activation and degranulation.

An enhanced level of circulating PR3 has many implications in the pathophysiology and perhaps also the pathogenesis of systemic vasculitis. A higher risk for local protease/antiprotease imbalance due to increased amounts of circulating ANCA antigen could be determined genetically [7,8]. It could also be acquired through, for instance, alpha1-antitrypsin inactivation or inhibition of PR3/alpha1-AT complex formation by anti-PR3 ANCA [4,5]. A PR3 receptor has recently been demonstrated on human umbilical vein endothelial cells. Increased uptake could induce increased IL-8 and MCP-1 production, recruiting neutrophils and monocytes, or even lead to endothelial cell apoptosis [29–31]. PR3 exerts feedback regulation on myeloid differentiation. A defect in the down-regulation of PR3 in the promyelocytic stage has been suggested to cause enhanced myeloid differentiation, which may lead to enhanced inflammation [2]. PR3 also has multiple effects on the modulation of inflammatory mediators. It can, for example, cleave TNF-alpha, IL-1β and IL-2R, cleave and inactivate C1 inhibitor and IL-6, activate latent TGFβ and cleave IL-8 to a more active form [9]. Taken together, these features might be important for the cytokine/chemokine balance at local foci of inflammation.

In conclusion, our data show significantly raised levels of PR3 in plasma from patients with systemic small vessel vasculitis, regardless of ANCA specificity. This was not due to decreased renal function, ongoing inflammation, neutrophil or mononuclear cell activation. Plausible mechanisms, which need further elucidation, include defects in the reticuloendothelial system, genetic factors, and selective neutrophil degranulation or leakage.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council (projects 3910, 71X-09487), the foundations of the Royal Physiographic Society and Thelma Zoega, the Foundation for Strategic Research and the Medical Faculty, Lund University. Thanks to Carina Linder, Lena Gunnarsson and Åsa Pettersson for their skilful laboratory assistance.

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