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The Role of Proteinase 3 and Neutrophils in ANCA-Associated Systemic Vasculitis

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1. Introduction

Systemic vasculitides are a group of disorders characterized by vascular inflammation, leading to vessel occlusion and consequent necrosis or ischemia. Depending on site and extent of inflammation, vasculitis has a varied presentation and prognosis. The classification of systemic vasculitides is based on the dominant vessel involved. They are also classified as idiopathic, primary and secondary to connective tissue diseases (rheumatoid arthritis, systemic lupus erythematosis), infections (infective endocarditis) and drugs (Firestein GS, 2008; Watts R, 1995). AASV (ANCA-Associated Systemic Vasculitis) is the most common primary small-vessel vasculitis that occurs in adults, and recent data indicates that the incidence has shown an up-swing. As per recent reports, the annual incidence of AASV varies from 12.4 to 19.8 per million. In two recent studies by our group, we found an incidence for AASV of 20.9/million, with a point prevalence of 268/million inhabitants in southern Sweden (Knight, 2006; Mohammad, 2007; Mohammad, 2009).

ANCA (anti neutrophil cytoplasmic antibodies)-associated Vasculitis is a term that refers to a group of disorders marked by multi-organ system involvement, small vessel vasculitis and the presence of ANCA. These include Wegener's granulomatosis (WG), Churg-Strauss syndrome (CSS) and Microscopic polyangiitis (MPA). The two most important ANCA antigens are PR3 and MPO. The vast majority of anti-PR3 antibodies yield a c-ANCA (cytoplasmic) pattern on IIF, while most anti- MPO antibodies produce a p-ANCA (perinuclear) pattern, with some exceptions (Segelmark, 1994). As per an international consensus document from 1999, anti-MPO and anti-PR3 antibodies are referred to as MPO-ANCA and PR3-ANCA. AASV is characterized histologically by leukocytoclasis, infiltration and accumulation of apoptotic and necrotic neutrophils in tissues, and fibrinoid necrosis of the vessel walls. The histological lesions in AASV are also termed pauci-immune, as only a few or no immunoglobulins/ complement components are detected in the vasculitic lesions. AASV is associated with significant morbidity and mortality (median survival of five months, in the absence of treatment), with almost all patients requiring long term and aggressive immunosuppressive treatment (Booth, 2003).

The etiology of AASV remains largely unknown. Genetic predisposition (PIZ allele of α1-AT, CTLA-4, PTPN22, HLA DR1-DQw1) and environmental factors including exposure to silica and asbestos, drugs (anti-thyroid medications), and various infections (bacterial endocarditis, hepatitis C virus) have been demonstrated to either predispose to, or correlate with ANCA and development of vasculitis (Beaudreuil, 2005; Choi, 2000; Elzouki, 1994;

Hay, 1991). The pathophysiological processes underlying the development of AASV are not fully understood; data suggests that neutrophils and lymphocytes play key roles. Intriguingly, though the association between ANCA and pauci-immune small vessel vasculitides is well established, the exact role of ANCA in the pathogenesis of AASV remains an enigma. This review focuses on the role of proteinase 3 and neutrophil apoptosis in AASV.

2. Proteinase 3

Proteinase 3 (PR3), also called myeloblastin and proteinase 4, was originally identified by Ohlsson, and later characterized by Baggiolini et al (Baggiolini, 1978; K. Ohlsson & I. Olsson 1973). PR3 is a neutral serine protease found in the azurophilic granules of neutrophils and peroxidase-positive lysosomes of monocyte. It is also present in specific granules and in secretory vesicles, and is expressed on the plasma membrane of normal blood neutrophils (Csernok, 1994; Rao, 1991; Witko-Sarsat, 1999). The PR3 gene maps to chromosome 19p13.3, in a cluster with HLE and azurocidin (AZU); it spans 6570 base pairs and consists of five exons and four introns (Sturrock, 1993). Introns I and IV include regions with repeating motifs, which may cause chromosomal instability and a predisposition to genetic rearrangements and deletions. A bi-allelic restriction fragment length polymorphism (RFLP) has been described in the PR3 gene. The gene is transcribed in the promyelocytic stage. Allelic variations in PR3 may be associated with quantitative/qualitative differences in the expression and/or function of PR3 (Gencik, 2000). PR3-mRNA is detected in early cells of the myeloid lineage and is down-regulated during myeloid differentiation. The mechanisms that promote high level transcription of PR3 in myeloid cells committed to granulocyte differentiation are not completely understood, although it is known that two transcriptional factors are needed for the expression of PR3, PU.1 and CG element (Sturrock, 1996).

PR3 is synthesized as a prepro-enzyme, which is processed in four consecutive steps into a mature form consisting of 222 amino acids. Following removal of signal peptide, it is transported into the endoplasmic reticulum (ER), where it is glycosylated with high-mannose oligosaccharides. Glycosylation of PR3 may influence its subcellular localization, with certain glycosylated isoforms being designated for granular cells and others for secretion or expression on the plasma membrane. The propeptide of PR3 is removed in the post-Golgi organelle, after which a seven-amino-acid carboxy-terminal extension is removed, possibly by a trypsin-like proteinase. During this process, small amounts of the pro-form of PR3 escape granular targeting and are secreted. These molecules may play a role in negative feedback regulation of granulopoiesis.

2.1 Membrane expression of PR3

PR3 is expressed on the plasma membrane (mPR3) of a subpopulation of resting neutrophils. Halbwachs-Mecarelli et al. noted the existence of two distinct neutrophil subpopulations, mPR3+ and mPR3-negative, in normal healthy individuals, termed as the bimodal expression of PR3 (Halbwachs-Mecarelli, 1995), Figure 1. Despite the high variability in the proportion of PR3-expressing cells among individuals, the proportion is highly stable in a given individual over long periods of time, suggesting genetic control of mPR3 expression (Schreiber, 2003). This is supported by twin studies demonstrating that the proportion of mPR3 expressing neutrophils in monozygotic twins is highly concordant. The intracellular levels of PR3 do not correlate with mPR3 levels. mPR3 is released from and

recruited to the plasma membrane on a continuous on- going basis, such that the amount of mPR3 on the surface of mPR3+ neutrophils remains relatively constant (Bauer, 2007).

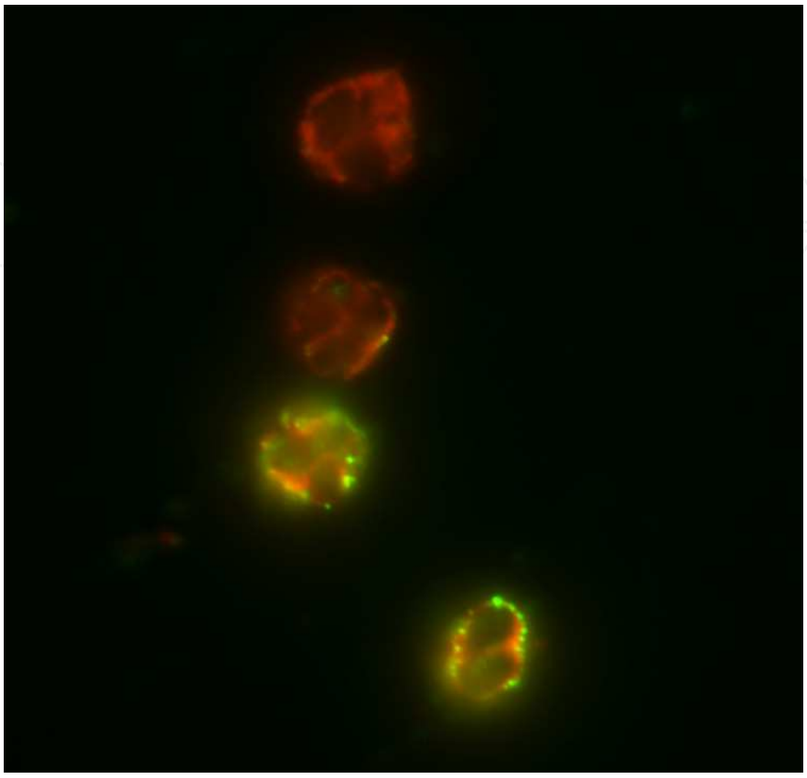

Fig. 1. Bimodal expression of PR3. A Fluorescent micrograph showing four neutrophils, the lower two cells express PR3 (represented with green colour, Alexa Fluor 488), while the upper two neutrophils do not express PR3 on their membrane. All neutrophils contain PR3 intracellularly shown in red (Alexa 594).

Expression of PR3 on the membrane of neutrophils is upregulated by multiple proinflammatory mediators including TNF-α, PMA, IL-18, LPS, IL-8, PAF, fMLP and GM-CSF, and by one anti-inflammatory cytokine: TGF-β (Campbell, 2000; Csernok, 1996; Hellmich, 2000). Membrane PR3 is active and quite resistant to inhibition by naturally occurring proteinase inhibitors including α1-AT, possibly due to steric hindrance of the membrane-embedded protease. PR3 can be eluted from the membrane of PMN following cellular activation; ionic interactions are important in the binding of PR3 to the plasma membrane. It is a cationic protein (isoelectric point 9.1), can bind stably to anionic and neutral membranes, but binds more strongly to negatively-charged bilayers. It has been suggested that PR3 membrane binding is possibly mediated by protein partners such as FcγRIIIb (CD16b), or β2 itegrin (CD11b/CD18). Fridlich et al. showed that cleavage of neutrophil glycosylphosphatidylinositol (GPI) anchors by phosphatidyl inositol-specific phospholipase C (PI-PLC) reduces the level of mPR3, indicating that a GPI protein, possibly FcγRIIIb, (or another yet unidentified GPI-anchored protein) attaches PR3 to the membrane (Fridlich, 2006). PR3 is also expressed on the plasma membrane of apoptotic cells, independent of degranulation, and this is associated with phosphatidylserine (PS) externalization. Kantari et al. demonstrated that phospholipid scramblase- 1 (PLSCR1)

interacts with PR3 and may promote its translocation to the plasma membrane during apoptosis (Kantari, 2007).

2.2 PR3 functions

PR3 is an autoantigen, possesses catalytic activity, is a hematopoietic regulator and has apoptosis inducing capabilities. mPR3 shows enzymatic activity; it degrades fibronectin, elastin, laminin, collagen type IV and heparan sulfate proteoglycans in the subendothelial matrix (Campbell, 2000). The soluble form of PR3 cleaves and activates cytokine precursors, including IL-8, IL-1β, and TNFα. PR3 also induces detachment and cytolysis of endothelial cells in vitro (Ballieux, 1994). A secreted proform of PR3 (retaining an amino terminal dipeptide) can downregulate DNA synthesis in normal CD34+ hematopoietic progenitor cells (S phase reduction); thus, PR3 may act as a negative feedback regulator of granulopoiesis in the bone marrow (Skold, 1999). Interestingly, this inhibitory effect of pro-PR3 is reversible; it can be abrogated by G-CSF or GM-CSF. PR3 actions are inhibited by α1-AT. MPO protects the enzymatic activity of PR3 by oxidizing a histidine residue on α1-AT, which tilts the protease anti-protease balance at sites of inflammation.

A recombinant cellular model has been used to demonstrate that PR3 plays a role in neutrophil survival. In particular, PR3 activates procaspase-3 into a specific 22-kDa fragment localized to the membrane compartment of neutrophils, but lacking from apoptotic neutrophils. This PR3-activated caspase-3 is restricted to the plasma membrane-enriched compartment, and segregated from its target proteins that mediate apoptosis from downstream components of the caspase-3 cleavage cascade. Thus in this model, PR3 can cause activation of caspase 3, but not apoptosis. Vong et al. devised a novel assay for PR3-protease activity using double-tagged recombinant annexin A1 (AnxA1) as substrate. This substrate was cleaved by recombinant PR3 or the membrane fraction of cells stably-transfected with PR3 in vitro and in vivo, suggesting that AnxA1 may be a physiologically-relevant substrate for PR3. AnxA1 has counter-regulatory inhibitory properties, and functions as an anti-inflammatory protein as well as inducer of neutrophil apoptosis. In activated neutrophils, AnxA1 translocates to the membrane, and becomes available for PR3. It is likely that cleavage of AnxA1 by PR3 decreases its innate inhibitory function, and promotes a pro-inflammatory response (Pederzoli, 2005; Vong, 2007). All these studies, together with the observation of high levels of PR3 within fibrinoid necrotic lesions in vasculitis, provide strong evidence that PR3 promotes a pro-inflammatory response.

2.3 mPR3 and CD177

CD177, also known as Polycythemia Vera protein-1 (PRV-1), is a glycoprotein that was first discovered in 1970 in connection with studies of Polycythemia Vera (Lalezari, 1971). It belongs to the Leukocyte Antigen 6 (Ly-6) supergene family and is the best characterized member of this family (Caruccio, 2006). As with PR3, CD177 has the unique distinction of being expressed on a subset/ fraction of the neutrophil population (Stroncek, 2004). In neutrophils that express CD177, CD177-mRNA levels are increased by exposure to G-CSF and by inflammatory states (sepsis, burns) associated with increased neutrophil production (Bux J, 2002; Gohring, 2004). CD177-mRNA is more abundant in CD177+ neutrophils than in CD177– PMNs (Wolff, 2003). Complete CD177-mRNA is not detected in CD177– neutrophils, suggesting a defect in transcription or splicing of CD177 mRNA. The functions of CD177 are not known, although there is evidence that it may play a role in adhesion of neutrophils to endothelial cells. CD177 can directly bind to PECAM-1 (CD31), expressed at

the junctions of the endothelial cells, on the membrane of neutrophils, monocytes and platelets, enhancing transendothelial migration of CD177+ neutrophils (Goldschmeding, 1992; Sachs, 2007). Also, CD177 is thought to be a marker of increased granulopoiesis.
We have shown that mPR3 and CD177 are co-expressed on the same subset of circulating neutrophils in AASV patients (Bauer, 2007), Figure 2. Also, we found that both CD177 and mPR3 are up-regulated in parallel, and to a similar extent, in this neutrophil subset. Following stimulation of cells with PMA or with CyB/fMLP, mPR3 and CD177 expression are co-induced approximately five-fold on the membrane of mPR3+/CD177+ cells and also converted the mPR3-negative/CD177-negative cells to mPR3/CD177-expressing cells, Figure 3.

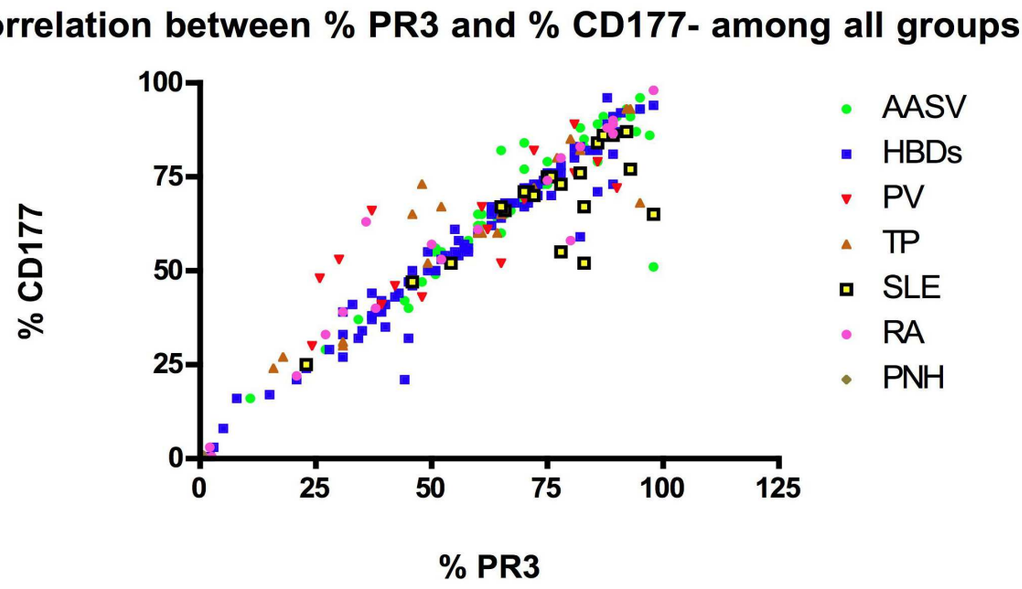

Fig. 2. Correlation between mPR3 and CD177 among all the groups. Shows the results of 91 HBDs, 52 AASV patients, 17 PV patients, 20 TP, 21 SLE patients, and 17 RA patients and one patient with PNH. There was a strong correlation between % of mPR3-positive neutrophils and % of CD177-positive neutrophils among all the groups, i.e. they define the same population of neutrophils (mPR3- and CD177-positive population). AASV= ANCA-associated Systemic Vasculitis. HBD= healthy blood donors. PV= Polycythemia Vera. TP= renal transplant recipients. SLE= Systemic Lupus Erythematosus. RA= Rheumatoid Arthritis. PNH= Paroxysmal Nocturnal Hemoglobinuria.

The bimodal expression of mPR3 in neutrophils is not explained by binding of mPR3 to CD16 and CD18 as CD16 and CD18 are expressed on all neutrophils. It may be that certain adaptor/transport proteins, possibly CD177 itself, that are expressed primarily in mPR3-positive cells, play a role in the expression of mPR3 on a subset of neutrophils. Von Vietinghoff et al. have provided evidence of direct binding between PR3 and CD177. Because the concentration of intracellular PR3 is similar in all cells, these putative adaptor proteins would be required to selectively facilitate PR3 localization to the plasma membrane in CD177-positive cells. It can also be postulated that a subset of cells, in which large amounts of PR3 and CD177 are stored in secondary and secretory vesicles during granulopoiesis, are precursors to the mPR3+/CD177+ circulating neutrophils. This would

argue for the existence of a genetic mechanism, whereby the genes encoding PR3 and CD177 are co-regulated during the later stages of granulopoiesis. The fact that only 4% of cells express only one of the two markers favours this hypothesis, and suggests that a similar mechanism is involved in mobilizing PR3 and CD177 from a common intracellular storage site to the plasma membrane (Bauer, 2007).

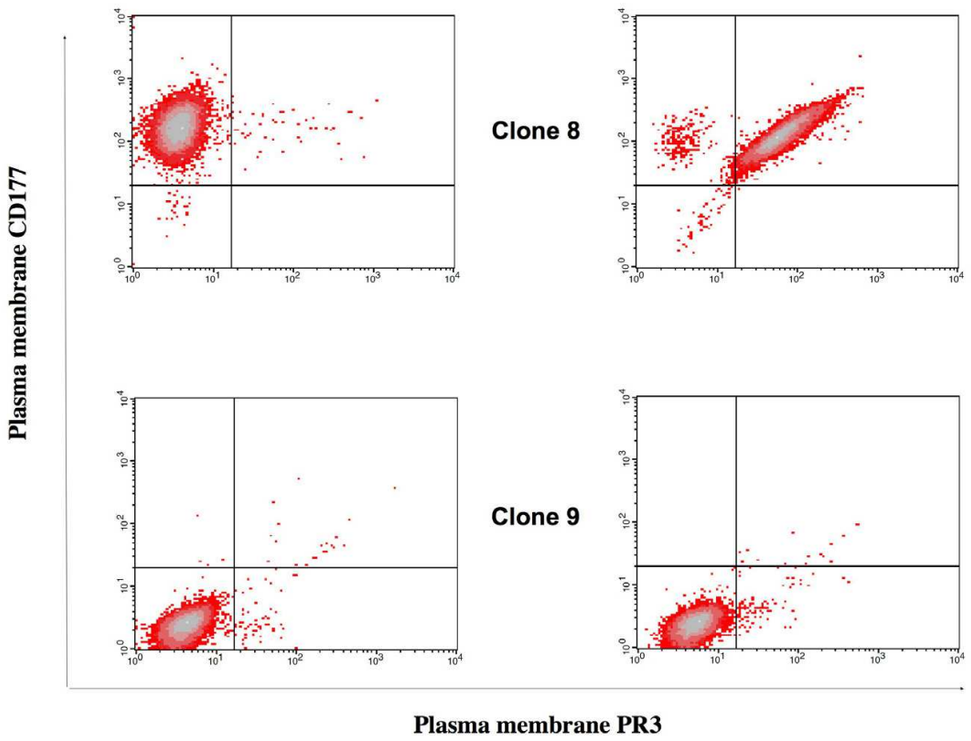

Fig. 3. U937 and exogenous PR3 binding. The left panel shows the membrane expression of U937-clone 8-cells (express CD177 but not PR3 on their plasma membrane), and U937-clone 9-cells (do not express PR3 or CD177 on their plasma membrane), measured by FACS. In the right panel, membrane expression of PR3 and CD177 was measured again on the same cells after incubation with mature PR3 for two hours. Clone 8 cells expressed the PR3 on their plasma membrane (upper right), while clone 9 cells did not express any PR3 or CD177 on their membranes (lower right).

2.4 PR3 and AASV

An A/G single nucleotide polymorphism (SNP) at coordinate -564 in the PR3 promoter has been identified, and it has been suggested that it was associated with WG. However, Pieters et al. showed that the -564 A/G polymorphism did not increase activity of the PR3 promoter, arguing against the possibility that the polymorphism results in an increased transcription/production of PR3 in WG patients (Pieters, 2004). In experiments performed by our group, the G allele of the -564 A/G polymorphism in the promoter of PR3 was not associated with WG (60% WG versus 69% HBD) or the $mPR3^{high}$ phenotype in AASV patients (Abdgawad, 2006). We did find a significant correlation between high plasma PR3 and the A allele of the -564 A/G polymorphism in HBD and in AASV patients, indicating

that the -564 A/G polymorphism might influence plasma PR3 levels.The fact that heterozygocity for deficiency alleles of α1-AT are associated with WG suggests that defects in the enzymatic function of PR3 may have functional effects. It has also been reported that presence of the PiZ allele correlates with poor prognosis (Segelmark, 1995).

Patients with systemic small vessel vasculitis exhibit higher plasma levels of PR3 than healthy persons and disease controls. This holds true also during stable remission and shows no relation to general inflammation, medical treatment or decreased renal function (Henshaw, 1994; Ohlsson, 2003). Studies by our group have shown that the levels of plasma PR3, mPR3 and pro-PR3 are all elevated in patients with AASV (Abdgawad, 2010). Also, it was observed that mPR3+ neutrophils are more abundant in AASV compared to healthy donors, which agrees with previous studies suggesting that a high percentage of mPR3+ cells may be a risk factor for vasculitis. Circulating neutrophils and monocytes from patients with AASV display upregulated transcription of the PR3 gene. It is likely that aberrant PR3/mPR3 expression may reflect, or be a marker of a specific functional defect in neutrophils. A possible origin of high plasma levels is shedding of membrane PR3. Witko-Sarsat et al. reported that the $mPR3^{high}$ phenotype was more frequent in vasculitis patients than in controls, independent of the ANCA antigen specificity (Witko-Sarsat, 1999). We have reported a weak but significant correlation between plasma PR3 and mPR3–MFI in MPO–ANCA-positive patients, which suggests that shedding of PR3 from the membrane may be at least partly responsible for increasing the plasma level of PR3. However, this correlation was not seen in PR3–ANCA patients. It is possible that PR3–ANCA either enhances clearance of plasma PR3 from the circulation, or interferes with detection of PR3 by ELISA. In support of this hypothesis, a significant negative correlation between plasma ANCA levels and plasma PR3 levels in the subgroup of PR3–ANCA patients was observed, while this was not seen in the MPO–ANCA patients.

Rarok et al. found that the length of time between diagnosis and relapse was significantly shorter in WG patients with high mPR3 expression (total level of mPR3 expression), and that individuals with high total mPR3 expression were more likely to have a relapse than patients with low mPR3 (Rarok, 2002). Csernok et al. showed that PR3 induces maturation of a fraction of blood monocyte derived dendritic cells (DC) in vitro (Csernok, 2006). In this context, they also observed that PR3 activates PAR-2 receptor-dependent signaling, which in turn up-regulates HLA-DR, CD80, CD83 and CD86 and down-regulates CD14. These PR3-activated DCs stimulate autoreactive Th1-type PR3-specific CD4+T cells.

Our group has demonstrated that the mPR3+/CD177+ neutrophil subpopulation was larger in AASV patients as compared to healthy controls, which suggests a distinct pathophysiological basis (Abdgawad, 2010). mPR3 and CD177 exhibit a parallel dynamic membrane expression with rapid internalization and re-expression. Interestingly, higher CD177–mRNA, but not PR3–mRNA was found to correlate with a higher proportion of mPR3+/CD177+ cells, suggesting that overproduction of CD177 could lead to an increase in the proportion of mPR3+/CD177+ neutrophils. Consistent with this, CD177 mRNA was significantly higher in mPR3- positive than in mPR3-negative human neutrophils, while PR3 mRNA was not.

3. Neutrophils and neutrophil apoptosis

Neutrophils represent 60 to 70% of the total circulating leukocytes and are the major phagocytes of the body's defense system against infections. Mature neutrophils are non-

proliferating, non-dividing cells with a segmented nucleus, mixed granular populations (staining pink or purple-blue following treatment with a neutral dye), small Golgi regions and accumulation of glycogen particles. On an average, a neutrophil contains 200 to 300 granules, one third of which are peroxidase positive (azurophilic), while the rest are peroxidase-negative (specific and tertiary). Azurophilic granules are spherical, appear at the pro-myelocytic stage and contain MPO, serine proteases and antibiotic proteins, and form the microbicidal compartment of neutrophils.

C/EBPα and PU.1 are both key regulators of granulopoiesis and myelopoiesis. Neutrophil development requires co-expression of C/EBPα and low amounts of PU.1 (Ward, 2000; Lenny, 1997). While GM-CSF is important for the growth of neutrophil progenitors in early stages, G-CSF is necessary for their terminal differentiation into mature neutrophilic granulocytes. G-CSF increases the rate of production of neutrophils by reducing their maturation time in bone marrow, while the half-life of circulating neutrophils is mainly unaffected. In contrast, GM-CSF markedly increases the half-life of the neutrophils in circulation, while the production rate is only slightly increased (Lord, 1992). Skold et al. have shown that a secreted proform of Proteinase 3 (PR3) acts as a negative feedback regulator of granulopoiesis, and counters the effect of G-CSF (Skold, 1999). It is interesting that this feedback regulation by PR3 is reversible and abrogated by GCSF and GM-CSF.

Neutrophils contribute to immune surveillance and participate in elimination of micro-organisms and cell debris. This major function of neutrophils can be divided into 5 step functions; (1) adhesion. (2) trans-endothelial migration/diapedesis, (3) Interstitial migration/locomotion, (4) phagocytosis of bacteria and/or degranulation, and (5) apoptosis. Neutrophils are activated via two steps, priming and full activation. Multiple agents including bacterial products, cytokines such as TNF-α, GM-CSF, IL-8 and IFN-γ can prime neutrophils. Neutrophils are then mobilized to the site of infection/ inflammation by the help of chemoattractants where they encounter a second stimulus by which they become fully activated and kill bacteria or ingest cell debris. Migration of neutrophils from the circulation to the site of infection/inflammation is controlled by interactions with the vascular endothelium. L-selectins expressed on neutrophils allow rolling and loose adhesion of neutrophils to ligands expressed on the endothelial cell membrane (like E- and P-selectins). This loose adhesion leads to conformational changes in the leukocyte integrins of the β2 subfamily (CD11a, CD11b, CD11c/CD18), leading to engagement of other adhesion molecules on the membrane of endothelial cells such as intercellular adhesion molecule-1 (ICAM-1), ICAM-2, vascular cell-adhesion molecule-1 (VCAM-1) and mucosal vascular cell adhesion molecule-1 (MDAM-1), leading to high affinity ligand binding and firm adherence (Ley, 2007). Then, binding of chemoattractants such as IL-8, released from the endothelial cells, to neutrophil receptors lead to arrest of the neutrophil rolling. At the site of infection, membrane receptors recognize and bind opsonized bacteria leading to the formation of pseudopodia, phagocytosis of the pathogen in a phagosome that fuses with protease-rich granules leading to the destruction of the pathogen within the intracellular phagosome. Neutrophil phagocytosis of bacteria and cell debris involves the Fcγ-Receptors (FcγRIIa/ CD32 and FcγRIIIb/ CD16) and the complement receptors (CR1/ CD35 and CR3 or CD11b/CD18 integrin) (Witko-Sarsat, 2000).

Neutrophils express an array of proteases, contained in their granules, and can generate reactive oxygen species (ROS) in order to rapidly kill phagocytosed bacteria (Spitznagel, 1990). Once activated, they attack the invading pathogens by a combination of three

mechanisms: phagocytosis, degranulation, and extracellular traps. During phagocytosis, the neutrophils ingest the pathogen forming a phagosome; while at the same time secrete ROS (reactive oxygen species) and hydrolytic enzymes to destroy it. Degranulation refers to the process by which various cytotoxic molecules residing in cytoplasmic granules are released. Examples include myeloperoxidase (MPO), an enzyme that is responsible for converting hydrogen peroxide to hypochlorous acid, a highly effective bactericide. Most recently, a novel extracellular mechanism (NETosis) of destroying pathogens has been described by Brinkmann et al (Brinkmann, 2004). Activation of neutrophils causes the release of chromatin fibers and granule proteins termed as neutrophil extracellular traps (NETs) that can trap and kill microbes extracellularly. NET formation is a part of active cell death; NETs are released when the activated neutrophils dies.

Neutrophils can also present antigens via MHC-II, thereby stimulating T cell activation and proliferation (Sandilands, 2005). Primed neutrophils actively synthesize and secrete cytokines, chemokines, leukotrienes and prostaglandins. In particular, neutrophils synthesize and secrete IL-8, IL-1, IL-1RA, IL-6, IL-12, TGF-β, and TNF-α (Cassatella, 1999; Fujishima, 1993). These cytokines can subsequently stimulate both neutrophils and other cells of the immune system. Neutrophils are significant source of leukotrienes and prostaglandins, especially leukotriene B4 (LTB4) and prostaglandin E2 (PGE2). PGE2 is an anti-inflammatory molecule, and has been reported to delay neutrophil apoptosis (Ottonello, 1998).

3.1 Neutrophils and AASV

The presence of activated neutrophils has been demonstrated at sites of injury in vasculitis lesions, both in lung infiltrates and renal biopsies (Brouwer, 1994; Travis, 1991). The number of activated neutrophils in renal biopsies correlated with extent of tissue damage. In a mouse model of MPO–ANCA associated vasculitis, neutrophil depletion reduced the number of vasculitic lesions(Xiao, 2005). In another Brown-Norway rat model of systemic necrotizing leukocytoclastic vasculitis induced by mercuric chloride and characterized by development of MPO-ANCA, a monoclonal antibody that depletes neutrophils could ameliorate vasculitis lesions (Qasim, 1996). These observations stand testimony to the key role of neutrophils in the pathogenesis of AASV.

The classical pathophysiological model of neutrophil activation can be divided into the following steps: an initial event (antigenic stimulus) primes neutrophils via cytokines (TNFα/IL-18/LPS) which subsequently induces membrane expression of PR3 and MPO. Priming induces the clustering of FcγRIIa and β2-integrins, formation of NADPH oxidase complex, increased expression of CD11b/CD18 and loose adherence of neutrophils to the endothelial cells. This is followed by binding of circulating ANCA to MPO and PR3 expressed on the neutrophil membrane. In the activation process, F(ab)´2 fragments of ANCA bind to their autoantigens, while Fc fragments bind to neutrophil Fcγ-receptors (FcγRIIa and FcγRIIIb) resulting in full activation of neutrophils (Falk, 1990).

There is substantial evidence for the activated state of neutrophils in AASV. In patients with active WG, neutrophils show increased expression of β1-(CD29), β2-(CD18) and α-(CD11b) integrin subunits (Haller, 1996). AASV neutrophils have been shown to have increased mPR3 expression and higher basal production of superoxide radicals. Alcorta et al have studied the leukocyte gene expression in ANCA positive vasculitis and showed >200 up-regulated genes, which correlated with disease activity (Alcorta, 2007). In normal situations,

the short-lived neutrophils die by apoptosis and are subsequently phagocytosed by macrophages. Circulating apoptotic neutrophils are cleared from circulation by macrophages located in the liver (29%), spleen (31%) and the bone marrow (32%) (Saverymuttu, 1985). Tissue neutrophils, which migrate to tissues during infections, are removed by local macrophages that secrete anti-inflammatory cytokines TGF-β and IL-10 upon phagocytosis of these neutrophils. For normal homeostasis to take place and in order to keep normal counts of neutrophils in the circulation (2.5-7.5 ×109/l), neutrophil turn-over must be tightly balanced between granulopoiesis and neutrophil apoptosis/clearance. Delayed neutrophil apoptosis has been associated with several acute and chronic inflammatory diseases (Simon, 2003).

3.2 Neutrophil apoptosis

Apoptosis, or "programmed" cell death, is a physiological form of cell death characterized by cell shrinkage, nuclear and chromatin condensation, DNA fragmentation, membrane blebbing, externalization of phosphatidylserine (PS), and formation of membrane-bound apoptotic bodies (Edwards, 2003). Many players are known to regulate apoptosis, including caspases, cell death receptors (of the TNF family), adaptor proteins, inhibitor of apoptosis (IAP) proteins and the bcl-2 family.

Neutrophil apoptosis occurs via the intrinsic or the extrinsic pathways. The intrinsic pathway is regulated by various proteins and molecules, including Mcl-1 and Bcl-2-A1 (Bfl 1) gene product and SHIP-1 (Edwards, 2003). Mitochondria play an important role in the intrinsic pathway of apoptosis through three key mitochondrial proteins; cytochrome c (cyt c), Smac/DIABLO and apoptosis inducing factor (AIF). The release of cyt c from the mitochondria is recognized as an initiator of apoptosis via interaction with Apaf-1 (apoptotic protease activating factor-1), leading to activation of caspase 9, formation of the apoptosome, and triggering of the caspase cascade. The Bcl-2 family regulates mitochondrial membrane permeability and cyt c release, thus playing a central role in apoptosis (Fossati, 2003). Neutrophils possess very few mitochondria and express low amounts of cyt c and Smac/ DIABLO, which are nonetheless sufficient to induce apoptosis. The tendency of neutrophils towards spontaneous apoptosis is inversely correlated with Bcl-2 expression.

The extrinsic pathway is initiated by an extracellular death signal. Death receptors bind to extrinsic factors (FasL, TNF-α, TRAIL) and activate the caspase cascade, which in turn generates intracellular death signals culminating in apoptosis. Death receptors such as Fas and the TNF receptor are integral membrane proteins. Fas and Fas ligand (FasL) interaction initiates apoptosis in a caspase-dependent manner. Neutrophils undergo spontaneous apoptosis more than other leukocytes, probably because they express both Fas and FasL on their plasma membrane (Edwards, 2003; Liles, 1996). Apoptosis-inducing factor (AIF) is a flavoprotein that is normally located in the inter-membrane space of mitochondria. When cells receive a signal for apoptosis, AIF is released from the mitochondria and translocates into the nucleus and causes nuclear fragmentation and cell death. The DNA destruction mediated by AIF is not blocked by caspase inhibitors and is thus considered a caspase-independent pathway. In neutrophils, AIF does not leave the mitochondria and the caspase-independent pathway is mediated by mitochondria-derived reactive oxygen species (ROS) (Edwards, 2003).

The mechanisms regulating spontaneous neutrophil apoptosis are not fully understood. Disturbance in the normal apoptotic process can enhance survival time, leading to a persistent inflammatory response. Blood neutrophils express fairly high levels of a range of pro-apoptotic proteins like Bad, Bax and Bik, but do not express the anti-apoptotic Bcl-2 and Bcl-xL proteins. Several pro-inflammatory agents, including IL-1β, L-2, IL-4, IL-6, IL-15, IFN- γ, G-CSF, GM-CSF and LPS, can delay neutrophil apoptosis (Simon, 2003). G-CSF induces survival of PMNs via the MEK-ERK pathway, leading to phosphorylation of Bad (inactivation) (Chuang, 1998). GM-CSF induces survival via the tyrosine kinase LynK-PI3K and JAK-2. G-CSF up-regulates the expression of Bcl-2-A1 and down-regulates the expression of Bax. GM-CSF up-regulates the expression of Mcl-1 and down-regulates the expression of Bax (Moulding, 2001). G-CSF, but not GM-CSF, selectively up-regulates the expression of cIAP-2, at the protein as well as mRNA levels. IAPs regulate apoptosis by binding to TNF-receptor associated factor-1 (TRAF-1)/ TRAF-2 heterocomplex to suppress activation of caspase 8. IAPs suppressing activation of caspase 9 and are capable of inhibiting the activation of caspases 3 and 7 directly (Edwards, 2003). TNF-α has a dual action on neutrophil apoptosis, leading to accelerated apoptosis in a susceptible subpopulation and delayed apoptosis in the surviving cells. TNF-α differential effects are dependant on concentration and the time of exposure. Adhesion of neutrophils to activated endothelial cells, IL-8, as well as transmigration of neutrophils through endothelial cell layer inhibits apoptosis.

During an inflammatory response, neutrophils produce numerous cytokines and chemokines, via up-regulation of gene expression. Once phagocytosis is accomplished, these functions are down-regulated in tandem with induction of apoptosis, leading to a decrease in pro-inflammatory capacity. This process is tightly regulated to prevent tissue damage caused by lingering neutrophils. Walcheck et al. have shown that phagocytosis-induced neutrophil apoptosis is accompanied by increase in the surface expression of ADAM17, followed by ADAM17-mediated release of IL-6R from cells, which then recruits mononuclear phagocytes to the site of infection (Walcheck, 2006). Recruitment of macrophages to sites of inflammation is also promoted by resolvins and protectins including lipoxin A4 (LXA4).The removal of apoptotic neutrophils is a non-phlogistic process, largely due to release of anti-inflammatory mediators.

3.3 Neutrophil apoptosis and AASV

Pathological specimens from patients of WG show clear presence of apoptotic and necrotic neutrophils (Travis, 1991). Leucocytes, with degraded nuclear material, undergoing disintegration and apoptotic cells have been observed in tissue specimens from ANCA-positive renal vasculitis (Rastaldi, 2000). Histologically, AASV is characterized by leukocytoclasis, with infiltration and accumulation of unscavenged apoptotic and necrotic neutrophils in tissues around blood vessels. E/M studies of the leukocytoclastic lesions, have suggested that there may be a defect in the clearance of apoptotic neutrophils. The minority of neutrophils in this study showed typical apoptotic changes of the condensed and marginated nuclei, while the majority showed intact nuclei with disintegrated cytoplasmic organelles and plasma membranes (Yamamoto, 2000). We have demonstrated significantly higher rate of survival and lower rate of apoptosis in AASV neutrophils as compared to neutrophils from healthy blood donors (HBDs) (Un-published data). It can be presumed that reduced apoptosis in AASV might be secondary to chronic inflammation.

However, the rate of apoptosis did not correlate with clinical parameters such as disease activity, CRP concentration, BVAS score or reduced GFR.

Interestingly, when neutrophils from AASV patients and HBDs were incubated with plasma from AASV patients, neutrophil survival was enhanced, suggesting that specific factors in the plasma influenced the apoptosis mechanism or rate. Growth factors are known to prolong survival by up-regulating anti-apoptotic factors and down-regulating pro-apoptotic factors. G-CSF, GM-CSF and LPS up-regulate expression of anti-apoptotic Bcl-2A1 and promote neutrophil survival, while Mcl-1 is up-regulated by GM-CSF, IL-1β and LPS. G-CSF up-regulates c-IAP2 (Inhibitor of Apoptosis Protein, IAP) (Santos-Beneit, 2000). IAP-2 is strongly up-regulated in mature neutrophils from patients with chronic neutrophilic leukemia, which also show prolonged in vitro survival. Christensson et al. showed that AASV patients in remission had higher circulating levels of soluble Fas than HBD and other disease controls (Christensson, 2002). No data from functional tests was available, and the effect of soluble Fas on Fas-mediated neutrophil apoptosis is not known. G-CSF, GM-CSF and IL-3 are known to enhance neutrophil survival, and delay or prevent neutrophil apoptosis. In our study, G-CSF and IL-3 levels were normal in plasma from AASV; GM-CSF level was higher than normal in four of 44 AASV patients. It is still possible that GM-CSF and IL-3 could be related to delayed apoptosis; neutrophils may have increased sensitivity to these cytokines. We tested this hypothesis and observed increased sensitivity in only three patients, who were more sensitive to GM-CSF/IL-3 than HBD. The proapoptotic factor Bax is down-regulated in response to G-CSF, GM-CSF, IL-3 and IFN-γ. Our group did not observe any correlation between the reduced rate of apoptosis or necrosis in AASV patients with higher levels of mRNA encoding these factors. A statistically insignificant increase in expression of Bcl-2A1 (1.45), Mcl-1 (1.78) and Bax (1.56) was noted in AASV neutrophils, compared to HBD neutrophils. Another possible mechanism of reduced apoptosis is alteration in neutrophil growth factor signaling. Our group has shown that the level of mRNA encoding three transcription factors, involved in steady-state and emergency granulopoiesis (C/EBP-α, C/EBP-β and PU.1), is significantly higher in AASV than in HBD (Un-published data). The target genes of these transcription factors include important neutrophil proteins including G-CSF receptor, GM-CSF receptor, myeloperoxidase, PR3, elastase, lysozyme and lactoferrin. It is possible that elevated expression of C/EBP-α, C/EBP-β and PU.1 in AASV neutrophils could lead to enhanced sensitivity to cytokines; a defect/deficiency of inhibitory factors may lead to perpetuation and exaggeration of survival signals and increased transcription factors.

Gilligan et al. showed that aging neutrophils (unprimed) were capable of translocating PR3 and MPO to the membrane during apoptosis, as assessed by increased ANCA binding (Gilligan, 1996). Another study showed that a small sub-fraction of TNFα-accelerated apoptotic neutrophils expressed higher levels of PR3 and MPO than TNFα-primed live neutrophils (Kettritz, 2002). Also, Kantari et al have shown that scramblase-1 translocates PR3 to the plasma membrane in a flip-flop manner during apoptosis (Kantari, 2007). In contrast, Yang et al demonstrated that the level of mPR3 is similar between apoptotic and non-apoptotic primed neutrophils (Yang, 2000). Our group has shown that though there is an increased fraction of neutrophils double-positive for membrane PR3 and CD177 in AASV (69% for AASV, 58% for HBD; p=0.004) expression, the percentage of double positive neutrophils does not correlate with the rate of neutrophil apoptosis, suggesting that membrane PR3 is not a pre-apoptotic marker (un-published data). Thus, although evidence

for increased membrane expression of auto-antigen in apoptotic neutrophils is inconclusive, it can be concluded that MPO and PR3 remain accessible for ANCA on the membrane of apoptotic neutrophils. Interestingly, Patry et al showed that injection of syngenic apoptotic neutrophils, but not freshly isolated neutrophils, into Brown Norway rats resulted in development of p-ANCA, with the majority being specific for elastase, again indicating that apoptotic neutrophils may boost an autoimmune response (Patry, 2001). Intraperitoneal infusion of live or apoptotic human neutrophils (but not formaline fixed or lysed neutrophils) into C57BL/6J mice resulted in development of ANCA specific for lactoferrin or myeloperoxidase. A second intravenous infusion of apoptotic neutrophils resulted in the development of PR3-specific ANCA. Again no vasculitic lesions were found in those mice developing ANCA.

ANCA themselves may dysregulate the process of neutrophil apoptosis. In an in vitro study conducted by Harper et al., ANCAs accelerated apoptosis of TNF-α-primed neutrophils by a mechanism dependent on NADPH oxidase and the generation of ROS (Harper, 2000). This was accompanied by uncoupling of the nuclear and cytoplasmic changes from the surface membrane changes. That is, while apoptosis progressed more rapidly, there was no corresponding change in the rate of externalization of PS (phosphatidyl serine) following activation of neutrophils by ANCAs. This dysregulation created a 'reduced window of opportunity' for phagocyte clearance by macrophages, leading to a more pro-inflammatory environment. It must be noted here that ANCAs were unable to accelerate apoptosis in unprimed neutrophils. Additionally, although there was increased expression of PR3 and MPO as apoptosis progressed, ANCAs were unable to activate these neutrophils. In fact, there was a time dependent decrease in ROS generation as these neutrophils aged. ANCA accelerates neutrophil apoptosis via generation of ROS, which act as amplifying factors for apoptosis. ROS are critical since neutrophils isolated from patients with chronic granulomatous disease (causing a defect in ROS production) do not show accelerated apoptosis after ANCA activation. The same authors, in a later study, as well as another independent group have shown that ANCA binding to apoptotic neutrophils enhanced phagocytosis by human monocyte-derived macrophages but also increased the secretion of pro-inflammatory cytokines like IL-1, IL-8 and TNF-α (Harper, 2000; Harper, 2001). IL-1 and IL-8 are capable of retarding apoptosis and are powerful chemoattractants. A pro-inflammatory neutrophil clearance will result in further cell recruitment and perpetuation of inflammation.

Apoptosis plays a crucial role in resolution of inflammation and maintaining self-tolerance. Defects in apoptotic pathways could potentially lead to the persistence of autoreactive T- or B-cells and contribute to development of autoimmune disease. Apoptotic neutrophils are a potential source of immunologically exposed neutrophil antigens that promote the production of ANCAs. From the available evidence, it may be inferred that there exists an altered neutrophil phenotype in AASV, which may be directly related to disease pathogenesis.

Enough evidence has accumulated for us to reasonably conclude that the neutrophils constitute two, molecularly well demarcated, sub-populations; one is positive for mPR3 and CD177 and the other subpopulation is negative for PR3 and CD177. The proportion of mPR3+/CD177+ cells is remarkably stable in a given individual, while a wide inter-individual variation can be observed. It is likely that these two subpopulations have distinct functions, which may have a direct bearing on pathophysiological processes. Membrane CD177 helps neutrophils adhere to the endothelium, while m-PR3 helps this positive

subpopulation to migrate through the endothelium and interstitial tissues. It may be inferred that the mPR3+/CD177+ cells possess greater killing capabilities, including higher NET and ROS production, than the mPR3–/CD177– sub-population. In simplistic terms, the mPR3+/CD177+ neutrophils may be the designated "fighting" neutrophils, designed to migrate from blood into tissues and promote pro-inflammatory, microbicidal functions, while mPR3-negative neutrophils are destined to stay in the intra-vascular compartment, until they are filtered by bone marrow, liver or pancreas and undergo apoptosis. Functional defects that lead to change in the proportion of mPR3+/CD177+ cells would, by default, promote a pro-inflammatory state. The elevated/ up-regulated transcription factors in patients of AASV, may potentially explain the increased PR3-mRNA expression and the decreased neutrophil apoptosis; decreased apoptosis rate as well as the elevated transcription factors provide indirect evidence for an altered neutrophil phenotype in AASV. Alteration in apoptosis and membrane expression of PR3/CD177 are clearly linked to the pathophysiology of this disease. Future studies must be aimed at elucidating the mechanisms underlying the altered neutrophil phenotype. Possible directions include: measurement of Fas in the plasma as well as membrane expression of Fas and Fas Ligand on neutrophils, evaluation of G-CSF receptor, GM-CSF receptor and IL-3 receptor over-expression by neutrophils, measurement of JAK-2 inhibition by measurement of SHIP-1, SOCS-1 and SOCS-3 in neutrophils. It may also be worthwhile to search for, hitherto unknown, exogenous survival factors in the plasma or endogenous survival factors inside the neutrophils. The significance of an altered neutrophil phenotype in AASV is certainly intriguing and, will hopefully stimulate detailed and quality research into its mechanisms and pathophysiological role.

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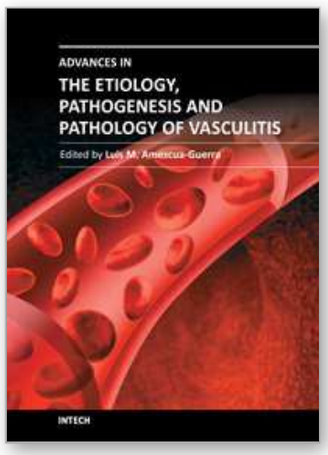

Advances in the Etiology, Pathogenesis and Pathology of Vasculitis

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This book represents the culmination of the efforts of a group of outstanding experts in vasculitis from all over the world, who have endeavored to devote their work to this book by keeping both the text and the accompanying figures and tables lucid and memorable. Here, you will find an amalgam between evidence-based medicine to one based on eminence, through an exciting combination of original contributions, structured reviews, overviews, state-of the-art articles, and even the proposal of novel pathogenetic models of disease. The book contains contributions on the etiology and pathology of vasculitis, the potential role of endothelial cells and cytokines in vascular damage and repair as well as summaries of the latest information on several primary and secondary vasculitis syndromes. It also covers selected topics such as organ-specific vasculitic involvement and quality of life issues in vasculitis. The editor and each of the authors invite you to share this journey through one of the most exciting fields of the medicine, the world of Vasculitis.

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