Neutrophil-endothelial interaction in ANCA associated vasculitis

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Chapter

8

Summary and general discussion

Summary

In this thesis, the interaction between neutrophils and endothelial cells in AAV was investigated, in particular the mechanisms containing neutrophils within the microvascular compartment in connection with the expression of PR3 on the neutrophil membrane.

The pathogenesis of AAV has not been fully understood, and it is intriguing that ANCA-associated vascular damage has a predilection for small-sized vessels. Capillaries and venules are the loci where leukocyte trafficking takes place during inflammation, and the endothelium of these vessels is particularly responsive to proinflammatory signals. There is no direct evidence that ANCA from AAV patients bind to and activate their own neutrophils in the circulation. In addition, ROS or degranulates released into the circulation by activated neutrophils will be rapidly diluted via blood flow or blocked by circulating inhibitors. So, these non-specific reagents may only damage the blood vessels when they get into close contact with vascular ECs. In **Chapter 2**, the interaction between neutrophils and endothelial cells in AAV and the effector mechanisms causing vascular damage were reviewed. We hypothesize that the synapse-like interaction formed between the neutrophil and the endothelial surface during adhesion or transmigration is the real battlefield for ANCA-activated neutrophils and the endothelium, and a prerequisite for the persistent inflammation in the vessel wall. Several factors, such as ANCA, proinflammatory and chemotactic cytokines are involved in this process. ANCA further activate neutrophils and cause release of ROS and proteolytic enzymes, which are concentrated close to adjacent endothelial cells by NETs and directly attack the vessel wall or activate endothelial cells resulting in more leukocyte recruitment. Activated endothelial cells are also actively involved in this sticky neutrophil-endothelial interaction by undergoing a protective mechanism against ROS and producing chemokines and cytokines. It is a complex process and involves many adhesion molecules, chemoattractants and their receptors, and immune modulators, which could offer plenty of opportunities for disease intervention.

We first tested the hypothesis that down-regulated expression of CXCR1/2 retains neutrophils within the vessel wall and, consequently, leads to persistence of neutrophils within the microvasculature. In **Chapter 3**, membrane expression of CXCR1 and CXCR2 on neutrophils was measured in a group of AAV patients in remission in comparison to HC. Serum levels of IL-8, TNF-α, ANGPT-1 and ANGPT-2 from quiescent and active AAV patients and HC were quantified.

Adhesion and transendothelial migration of isolated neutrophils was analyzed, with and without blockade of CXCR1 and CXCR2. Expression of CXCR1 and CXCR2 on neutrophils was significantly decreased in AAV compared to HC. Levels of IL-8, which dose-dependently down-regulated CXCR1 and CXCR2 expression on neutrophils in vitro, were significantly increased in the serum of patients with active AAV and correlated negatively with CXCR1/CXCR2 expression on neutrophils, even in quiescent patients. Blocking CXCR1 and CXCR2 with repertaxin, a specific inhibitor of these chemokine receptors, increased neutrophil adhesion and inhibited migration through a glomerular endothelial cell layer. It can be speculated that, in vivo, circulating IL-8 produced by activated endothelial cells or ANCA-activated neutrophils leads to decreased CXCR1 and CXCR2 expression on neutrophils, which, in turn, show increased adhesion and deficiency in transendothelial migration. Neutrophils accumulating in the microvascular compartment are subsequently activated by ANCA, and released ROS and proteolytic enzymes cause vessel damage.

As for the effector mechanisms causing damage to the vessel wall in AAV, the role of AECA is interesting but has been poorly analyzed. The presence of AECA in AAV has been reported by several groups with conflicting data regarding their prevalence ranging from 8% to 100% in AAV patients. Increased binding of AECA to endothelial cells isolated from nose, kidney and lungs, which are the most frequently involved organs in AAV, was demonstrated. These results suggest that AECA in AAV patients are organ-specific and could imply that, as substrates, endothelial cells from relevant organs should be used in AECA detection. Therefore, in **Chapter** 4, we investigated the prevalence of AECA in AAV using a human glomerular endothelial cell (GEnC) line in comparison with primary human umbilical vein endothelial cells (HUVEC), which are frequently used for AECA detection. As AECA might induce endothelial activation, serum levels of adhesion molecules, markers of endothelial activation, were also analyzed in a group of AAV patients. Generally, AECA had low frequency in AAV patients. AECA were detected in 4 of 29 WG patients (14%) and in none of 14 MPA patients using conditionally immortalized GEnCs as substrate, whereas AECA were positive in 10% of WG patients and 14% of MPA patients on HUVEC. No significant difference in OD value was found between AAV patients and controls in AECA testing. Serum levels of soluble VCAM-1 and ICAM-1 in AAV patients were significantly higher than in controls. However, no correlation was found between AECA titers and levels of soluble adhesion molecules and there were no differences between AECA-positive and -negative patients for both activation

markers. Theoretically, it could be helpful to use more than one type of substrate cells for AECA testing in order to increase their detection rate. However, the pathogenic relevance of AECA is doubtful, and elucidating the antigens of AECA in AAV is a prerequisite for further assessing their diagnostic and pathogenic role.

Membrane expression of ANCA-antigens, such as PR3, allows ANCA binding, is a crucial step in ANCA-mediated neutrophil activation, and has been shown to be significantly up-regulated during neutrophil adhesion. The PR3 molecule does not contain a transmembrane domain in its sequence, The mechanisms of membrane expression of PR3 and the signal transduction events following ANCA binding, therefore, become interesting and are reviewed in **Chapter** 5. PR3 is differentially expressed on the neutrophil membrane. The percentage of neutrophils with high levels of mPR3 expression ranges from 0 to 100%, and is rather constant within a given individual. CD177 is the receptor of mPR3 accounting for a substantial expression of mPR3 on the neutrophil membrane. On this $CD177^{+}$ subset of neutrophils, a complex of molecules colocalizes with PR3 on the neutrophil membrane and appears to be involved in signal transduction, including CD177, FcγRIIIb and β2-integrins. This complex probably functions in neutrophil recruitment, bringing these effector cells close to the endothelium and further mediates PR3-ANCA induced neutrophil activation. However, slight expression of mPR3 on $CD177^{-}$ neutrophils can also be detected, suggesting that CD177 is not an exclusive binding partner of mPR3. Other possible binding site(s) for PR3 and mechanism(s) involved in signal transduction, such as chondroitin sulfate- and heparin sulfate- containing proteoglycans, PLSCR1 and hydrophobic insertion, need further investigation. Possibly, these various molecules allow the two subsets of neutrophils to be equally involved in the pathophysiology of PR3-ANCA associated vasculitis regardless of CD177 expression.

The percentage of $mPR3^{high}$ neutrophils is increased in AAV patients and is a risk factor for relapse of GPA. Whether CD177 expression is also increased in AAV and responsible for mPR3 up-regulation, and what the role of CD177 is in PR3-ANCA-mediated neutrophil activation in AAV was investigated in **Chapter** 6. Expression of CD177 and mPR3 was analyzed in parallel on isolated neutrophils from patients with AAV, SLE, or RA, and healthy controls. Neutrophil activation mediated by anti-PR3 antibodies was assessed by measuring the oxidative burst. Percentages of $CD177^{+}$ neutrophils were significantly higher in patients with AAV and SLE compared to healthy controls. In 3 healthy donors, CD177 expression was not

detected. After priming with TNF-α, neutrophils from these 3 donors remained negative for CD177 while mPR3 expression was induced. Neutrophils from $CD177^-$ donors or $CD177^-$ neutrophils sorted from donors with bimodal expression were susceptible to anti-PR3–mediated oxidative burst. Variation in the extent of anti-PR3–mediated neutrophil activation among different donors occurred independent of the percentage of CD177-expressing neutrophils. These data confirmed our hypothesis that CD177-independent mPR3 expression does exist and both $CD177^+$ and $CD177^-$ neutrophils are susceptible for PR3-ANCA-mediated neutrophil activation.

Next, we studied differences between neutrophil subsets with and without CD177 expression. The molecular function of CD177 is largely unknown, except that it is a counterpart of PECAM-1 on endothelial cells, suggesting a role in neutrophil recruitment to the microvasculature. However, we did not observe significant differences in neutrophil adhesion or migration through GEnC monolayers between these two subsets (data not included in this thesis). Therefore, a microarray-based study was performed (**Chapter** 7). Differentially expressed genes between $CD177^+$ and $CD177^-$ neutrophil subsets might indicate differences in function. Gene expression in neutrophils was compared among donors with varying levels of CD177 expression, and between $CD177^+$ and $CD177^-$ subpopulations from donors with bimodal expression of CD177. A number of neutrophil granule proteins (GP), such as defensin α1, α3 and α4, NGAL, BPI or cathepsin G, which decline during neutrophil maturation, showed higher mRNA expression levels in the $CD177^-$ neutrophil subset in healthy donors, suggesting a link between $CD177^-$ and immature neutrophils. Indeed, by FACS analysis, we observed that CD177 expression emerges during neutrophil development. Therefore, differential expression of GP-related genes between $CD177^+$ and $CD177^-$ neutrophil subsets might reflect a different state of neutrophil maturation or different levels of enrichment with immature neutrophils. The amounts of these granule proteins stored in neutrophils, however, were comparable between $CD177^+$ and $CD177^-$ subsets; thus, functional differences at the protein level were not observed. Interestingly, up-regulation of these GP genes was also observed in AAV patients, which was unlikely to be related to a disturbed balance of the $CD177^+$ and $CD177^-$ subsets or an enrichment of immature neutrophils. Induction of GP gene expression in neutrophils by PMA or LPS suggests that on-going inflammation in AAV patients might be the reason for up-regulation of genes encoding granule proteins.

General discussion

It is an important feature of AAV that necrotic lesions, with paucity of immune complex deposition, occur preferentially in small-sized vessels. Neutrophils are the cells expressing ANCA antigens and also the cells producing ROS and proteolytic enzymes, which induce necrosis of endothelial cells. Early infiltration of large amounts of neutrophils in the target vasculature suggests that neutrophil accumulation is a sign of an upcoming vascular lesion and a prerequisite of ANCA-mediated vascular damage. It has been suggested that circulating neutrophils are unlikely to be activated by ANCA and to undergo the respiratory burst and degranulation, while adherent neutrophils attack endothelial cells. Neutrophil recruitment is a cascade process. Once triggered, rolling neutrophils will adhere to and subsequently migrate through the endothelium. Following these cascades, one would expect ANCA-medicated tissue damage to occur not only in the vessel wall but also in the interstitial tissues, which is obviously not the case. Careful study of renal biopsies from AAV patients revealed that neutrophils are accumulated in the glomeruli but poorly penetrate into the interstitial tissue where neutrophil chemoattractants were detected. Taken together, it is likely that neutrophil trafficking is triggered in AAV but it is stuck in the middle of the process by certain mechanisms. Therefore, answering the questions which mechanisms hamper neutrophil recruitment and what exactly happens during neutrophil-endothelial cell interaction are essential for understanding disease pathogenesis and for the development of novel therapeutic strategies.

Dysregulated neutrophil-endothelial interaction in AAV involves ANCA and proinflammatory cytokines in the circulation, which stimulate both neutrophils and endothelial cells leading to up-regulation or activation of adhesion molecules. In the current thesis, a chemokine-based mechanism is highlighted. CXCR1 and CXCR2 expression is significantly decreased on neutrophils from AAV patients and this decrease seems to be correlating with disease activity. Circulating IL-8, which shows increased levels in AAV, may be one of the factors inducing decreased expression of these chemokine receptors. As a consequence, neutrophils with decreased expression of CXCR1 and CXCR2 display increased adhesion and decreased migration through glomerular endothelium. These data suggest a relationship between circulating IL-8, chemokine receptors and neutrophil accumulation in the vessel wall. However, the role of CXCR1/2 in neutrophil adhesion has not been fully explained, as it has been shown that CXCR2 activation transduces a signal leading to firm adhesion. Therefore, the

detailed mechanisms of increased adhesion of CXCR1/2 deficient neutrophils deserves further investigation. Particularly, the adhesion molecules involved in this process and the structural or functional features of glomerular endothelial cells leading to these consequences are of great interest.

The effector mechanisms causing vascular damage are mainly ANCA-associated events. ANCA activate primed neutrophils leading to release of ROS and proteolytic enzymes, which directly attack the vessel wall and stimulate endothelial cells to recruit more inflammatory cells. NETs are formed by activated neutrophils. This structure may concentrate PR3 and MPO on the neutrophil surface for ANCA recognition and attacking of endothelial cells. AECA have been documented in AAV. However, their specific antigen(s) on endothelial cells have not been clarified so far. Our study on AECA in this thesis does not support a pathogenic role for AECA, but lets the possibility open that planted ANCA antigens on endothelial cells mediate ANCA-induced necrotic damage.

ANCA recognition of PR3 or MPO on the neutrophil membrane is a prerequisite for neutrophil activation. Membrane expression of these ANCA antigens is inducible by proinflammatory cytokines, such as TNF-α and IL-1β, and mPR3 expression is remarkably up-regulated during neutrophil adhesion. CD177 has been demonstrated to be a receptor of mPR3 on neutrophils. However, the detailed mechanisms of the physical interaction of these two molecules are not clear. As CD177 is possibly an adhesion molecule, and β2-integrins, key adhesion molecules on neutrophils, are colocalized with mPR3 in lipid rafts and mediate signals into the cell upon PR3-ANCA ligation, membrane expression of PR3 is no longer an event independent from neutrophil-endothelial interaction. In this context, a complex of molecules that colocalize with PR3, including CD177 and β2-integrins, on the neutrophil membrane appears to be involved. This complex probably functions in neutrophil recruitment by bringing these effector cells close to the endothelium and further mediating PR3-ANCA induced neutrophil activation and vessel damage.
We showed that the percentage of $CD177^{+}$ neutrophils is increased in AAV, which accounts for an enlarged subset of neutrophils with $mPR3^{high}$ expression. This skewed distribution could be genetically determined. However, increased $CD177^{+}$ neutrophil proportions have been observed in some other pathological conditions, such as severe infection, G-CSF treatment or pregnancy, suggesting that other mechanism(s) exist modulating the balance between these neutrophil subsets. This mechanism may become self-evident when the basic question is answered

why CD177 is differentially expressed in neutrophils. Functional consequences of increased CD177/mPR3 expression in AAV depend on the molecular function of CD177 and its implication on differences in function, if any, between $CD177^+$ and $CD177^-$ neutrophil subsets. The ability of CD177 to bind to PECAM-1 does not result in differences in adhesion or migration between these two subsets. Neutrophils are equally activated by PR3-ANCA, regardless of CD177 expression, suggesting that CD177 is not involved in the signaling pathway upon PR3-ANCA ligation. Finally, whole genome-wide analysis of gene expression revealed that granule protein-related genes are expressed at higher levels in the $CD177^-$ than in the $CD177^+$ neutrophil subset. This observation can be a departure point for further disclosure of the characteristics of these neutrophil subsets and the pathological role of this molecule in AAV.

In **conclusion**, the interaction between neutrophils and endothelial cells is crucial for disease development and intervention. Decreased expression of CXCR1 and CXCR2 on neutrophils in AAV is instrumental in neutrophil accumulation in the microvascular compartment. Effector mechanisms become operational in this process, but the role of AECA is minor. Membrane expression of ANCA-antigens, such as PR3, is induced during neutrophil recruitment. CD177 is responsible for mPR3 expression, but it is not essential for PR3-ANCA-mediated neutrophil activation. The underlying mechanism of the increased percentage of $CD177^+/PR3^{high}$ neutrophils in AAV is not clear. Differences in gene expression between $CD177^+$ and $CD177^-$ subsets cannot easily be translated into functional differences, but indicate heterogeneity of neutrophil populations. This heterogeneity is dysbalanced in AAV, but its exact pathophysiological role awaits further studies.