Increased levels of BLyS and sVCAM-1 in anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitides (AAV)

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ABSTRACT

Objective. *Anti-neutrophil antibodies (ANCA)-associated vasculitides (AAV) comprise different forms of small vessel vasculitis characterised by B-cell driven autoimmune processes and endothelial cell activation. Aim of this study was to correlate markers of B- and endothelial cell activation with clinical manifestations of disease in AAV.*

Methods. *Consecutive serum samples of patients fulfilling the Chapel Hill Consensus Conference (CHCC) and American College of Rheumatology (ACR) criteria for AAV and healthy donors were used for the determination of ANCA, B-lymphocyte stimulator (BLyS), soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble E-selectin (sE-selectin) levels using enzyme-linked immunosorbent assay (ELISA). Subset and follow-up analyses were performed in cytoplasmatic ANCA (C-ANCA) or perinuclear ANCA (P-ANCA) positive patients with respect to change in ANCA-titres during the course of disease.*

Results. *Levels of sVCAM-1 were elevated in all patient groups with vasculitis compared to healthy controls. In contrast, significantly increased levels of BLyS were only observed in patients with Wegener's granulomatosis (WG), but not in patients with microscopic polyangiitis (mPAN)/Churg-Strauss-syndrome (CSS). Remarkably, there were no differences in the levels of sE-selectin between the vasculitis groups and healthy controls. In follow-up analysis, a significant correlation was shown for sE-Selectin and P-ANCA titres as well as sVCAM-1 levels. Furthermore, a strong correlation was detected for sVCAM-1 and creatinine levels. Interestingly, sE-selectin levels and C-ANCA titres were negatively correlated.*

Conclusion. *Enhanced levels of sVCAM-1 represent a marker for endothelial cell activation in AAV. The observed correlation between sVCAM-1 and creatinine levels might indicate the influence of the vasculitic process on renal function. Signalling pathways for B-cells provided by BLyS could play a significant role in the pathogenesis of WG.*

Introduction

According to the Chapel Hill Consensus Conference (CHCC) the anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitidis (AAV) are classified into Wegener's granulomatosis (WG), microscopic polyangiitis (mPAN) and Churg-Strauss syndrome (CSS) (1, 2). These potentially life-threatening diseases are associated with high mortality (3) caused by necrotising small vessel vasculitis in various organs including lungs and kidneys.

As shown in animal and *in vitro* studies, ANCA play an important role in the pathogenesis of AAV (4). The major ANCA target antigens, myeloperoxidase (MPO-ANCA or P-ANCA) (5) and proteinase 3 (PR3-ANCA or C-ANCA) (6, 7) are physiologically expressed in the cytoplasm but also on the surface of a subset of neutrophils. It was shown that ANCA are capable to activate neutrophils and to promote their adhesion (8) and transmigration on endothelial cells *in vitro* (9). Furthermore, ANCA also induce expression of pro-inflammatory cytokines and toxic factors including MPO and PR3 by neutrophils (10). In return, the expression of MPO and PR3 is up-regulated by pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α. Taken together, growing evidence supports a role for ANCA in the inflammatory process, vascular injury and necrosis of endothelial cells in AAV (11, 12).

Although B-cell activation and the production of ANCA are characteristic features of AAV, the influence of

signalling pathways for B-cells was not analysed in detail so far. The B lymphocyte stimulator (BLyS), also known as BAFF, THANK, TALL-1, and zTNF4 is part of the TNF-family of ligands (13) and is expressed by monocytes, macrophages, dendritic cells and neutrophils (13-15). BLyS is a relevant factor for the development and lifetime of B-cells suppressing apoptosis and providing growth factor signals to B-cells with stimulation of antibody production (16). In animal studies it was shown that BLyS overexpression can induce a systemic autoimmune response (17). In this context, elevated levels of BLyS were observed in human autoimmune diseases such as rheumatoid arthritis (RA) (18, 19) and primary Sjögren's syndrome (pSS) (20). Furthermore, a correlation was shown between BLyS and autoantibody levels as well as disease activity in systemic lupus erythematosus (SLE) (21, 22). Therefore, in this study we investigated whether increased BLyS levels are associated with clinical manifestations of AAV.

The development of vascular injury and necrosis is dependent on increased adhesion and migration of leukocytes to endothelium. The first part of this interaction is mediated by selectins (E-, P- and L-selectin), followed by an interaction with immunglobuline-cell adhesion molecule superfamily receptors (ICAM-1 and VCAM-1) allowing adhesion of leukocytes to the endothelium and subsequently migration and extravasation. Increased levels of soluble E-selectin were observed in several autoimmune disorders, such as SLE and RA showing a correlation to disease activity and prognosis (23, 24). Another study documented high levels of soluble E-selectin in AAV at the time of initial diagnosis (25). Elevated levels of soluble VCAM-1 were also detected at initial diagnosis showing decrease in the remission phase in patients with AAV (25). Since the interaction between leukocyte surface receptors and their endothelial ligands might play a key role in the pathogenesis of AAV, the expression of sE-selectin and sVCAM-1 was analysed in more detail in this study.

Patients and methods

Consecutive serum samples of patients fulfilling the CHCC- (1, 2) and ACR-criteria (26, 27) for AAV and of healthy donors were used for determination of ANCA, BLyS, sVCAM and sE-selectin using ELISA. For the diagnosis of AAV a suitable clinical presentation provided by either positive ANCA serological findings or confirmed histological findings or both was required.

The analysed patients were divided into two groups, disease primarily associated with C-ANCA (WG n=41, 21 male and 20 female, mean age of 54.34±17.67 years) or P-ANCA (mPAN n=16 and CSS n=4, 9 male and 11 female, mean age of 57.1±20.11 years). Healthy donors served as controls (n=40).

Follow-up analysis (mean of follow up was 15 month) from the time point of diagnosis on were performed in 16 patients (WG n= 14, mPAN/CSS n=2; at least 5-7 serum samples per patient). The study was approved by the local Ethics committee and written informed consent was taken from each patient.

Measurement of ANCA

Serum samples were tested for the presence of C-ANCA and P-ANCA using ELISA according to the manufacturer's instructions (ORGENTEC Diagnostika GmbH, Mainz, Germany). The measurements were performed in duplicate.

ELISA for BLyS, sVCAM-1 and sE-selectin

BLyS, sVCAM-1 and sE-Selectin levels were analysed using commercially available sandwich ELISAs according to manufacturer's instructions (R&D Systems, Abingdon, UK). All measurements were performed in duplicate.

Measurement of CRP

Serum C-reactive protein (CRP) levels were measured by standard nephelometry. Values <0.5 mg/dl were considered normal.

Statistical analysis

All analyses were performed using SAS version 9.1.3 (SAS Institute Inc. 2004. SAS/STAT® 9.1 User's Guide. Cary, NC: SAS Institute Inc.). Means, standard deviations, and *p*-values were calculated by SAS procedures MEANS and Welch-Test. For correlation analysis we used the Pearson's correlation coefficient. For all tests, *p*-values less than 0.05 were considered as statistically significant.

Results

Levels of soluble adhesion molecules in AAV

The soluble forms of the adhesion molecules VCAM-1 and E-selectin were measured as markers for endothelial cell activation. As a result, significantly increased levels of sVCAM-1 were observed in patients with AAV compared to healthy donors (WG 716.46±350.49 ng/ml *vs*. healthy 459.87±126.20 ng/ml, $p<0.001$ and mPAN/CSS 906.97±416.71 ng/ml *vs*. healthy, $p=0.0002$) (Fig. 1 A, B). The measured levels for sVCAM-1 were not significantly different between patients with WG and mPAN/CSS ($p=0.0939$). Furthermore, there were no significant differences for sE-selectin in patients with AAV compared to healthy donors (data not shown).

Expression of BLyS in AAV

Since AAV are considered to represent B-cell driven autoimmune diseases, levels of the B-cell growth factor BLyS were determined as marker for B-cell activation. Interestingly, significantly increased levels of BLyS were only detected in the patients with WG compared to healthy controls (WG 1653.49±1097.20 pg/ml *vs*. healthy 968.38±274.68, $p=0.0003$) (Fig. 2). Levels of BLyS were not significantly different in patients with mPAN/CSS compared to healthy controls (mPAN/CSS 1770.54±1775.85 pg/ml vs. healthy, $p=0.0656$). Moreover, there was also no difference of BLyS levels in patients with WG compared to mPAN/CSS ($p=0.7933$).

Follow-up and correlation analyses of ANCA, levels of adhesion molecules and BLyS

Follow-up analyses were performed in 16 patients with AAV (WG n=14, mPAN/CSS n=2) from initial diagnosis over 5 to 7 different time points during course of disease. The parameters C-ANCA, P-ANCA, BLyS, sE-selectin,

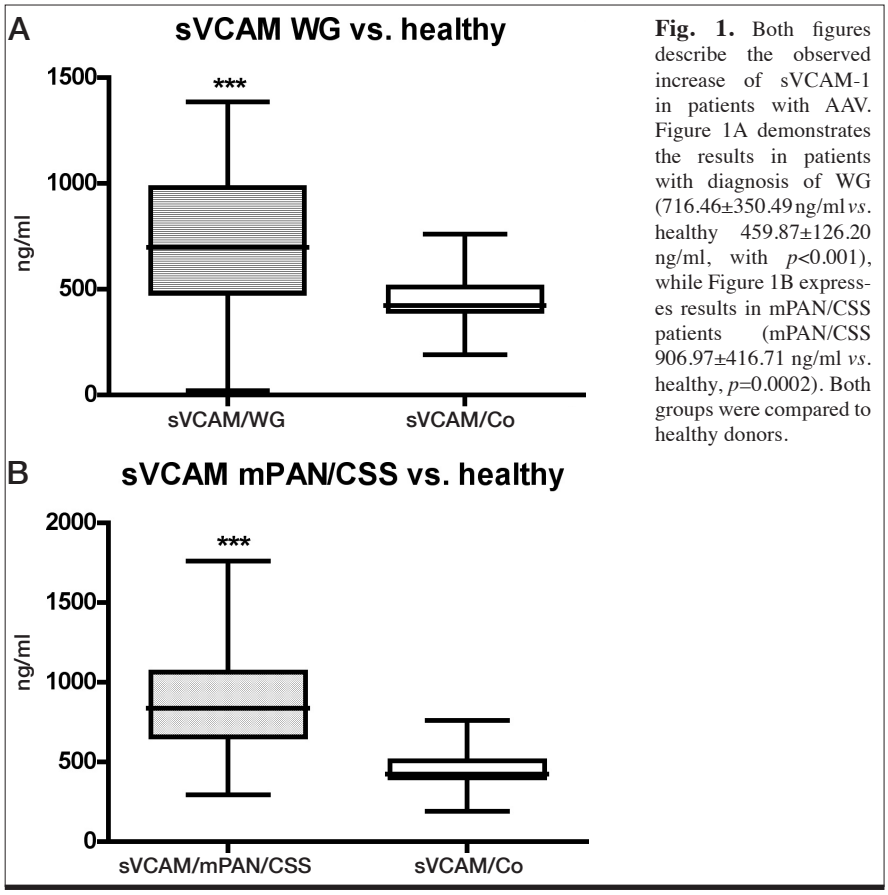

Fig. 1. Both figures describe the observed increase of sVCAM-1 in patients with AAV. Figure 1A demonstrates the results in patients with diagnosis of WG (716.46±350.49 ng/ml *vs.* healthy 459.87±126.20 ng/ml, with $p<0.001$), while Figure 1B expresses results in mPAN/CSS patients (mPAN/CSS 906.97±416.71 ng/ml *vs.* healthy, $p=0.0002$). Both groups were compared to healthy donors.

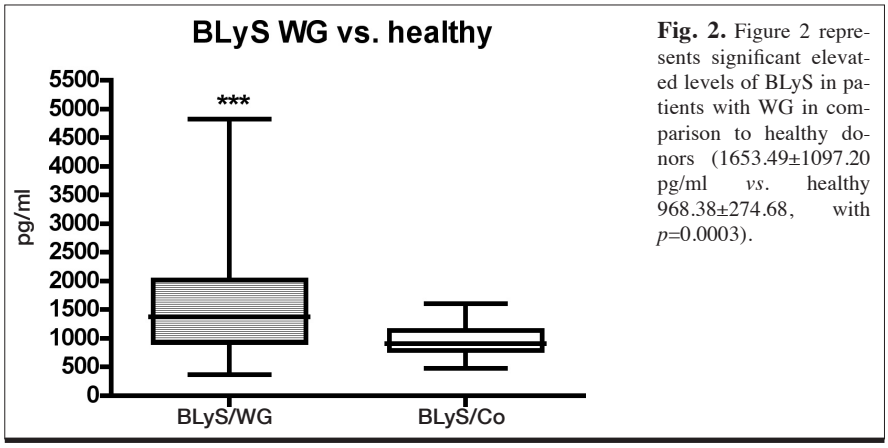

Fig. 2. Figure 2 represents significant elevated levels of BLyS in patients with WG in comparison to healthy donors (1653.49±1097.20 pg/ml *vs.* healthy 968.38±274.68, with $p=0.0003$).

sVCAM-1, creatinine and CRP were examined in order to detect possible correlations. Interestingly, levels of sVCAM-1 showed a strong correlation to creatinine values (r=0.82, $p=0.0001$).

As another result, a significant correlation was registered between sE-selectin levels and P-ANCA titres (r=0.60, $p=0.0137$). Furthermore, sE-selectin levels showed a negative correlation with c-ANCA titres (r=-0.57; $p=0.0211$) and a correlation to levels of sVCAM-1 (r=0.50, $p=0.0497$). Interestingly, levels of sVCAM-1 showed a strong correlation to creatinine values (r=0.82, $p=0.0001$).

A significant negative correlation was noticed between C-ANCA and P-ANCA titres (r=-0.59071; $p=0.0160$) (Table I). No further positive or negative correlations were found.

Discussion

In this study, we characterised markers for endothelial and B-cell activation in patients with AAV. As a result of endothelial activation, analyses of soluble adhesion molecules revealed significantly elevated levels of sVCAM-1 in patients with AAV including WG, mPAN and CSS. This finding is consistent with the known expression of VCAM-1 on activated endothelial cells and its involvement in the endothelial inflammatory processes (28, 29). Since increased levels of sVCAM-1 were observed in all investigated manifestations of AAV this marker seems to represent a rather non-specific phenomenon in systemic vasculitis.

Ara *et al*. (25) observed increased levels of sVCAM-1, sE-selectin and other adhesion molecules in ANCA-positive, small vessel vasculitis at the time of initial diagnosis. Furthermore, it was also shown that levels of certain adhesion molecules decrease during remission of disease. However, our study did not show a significant difference between levels of sE-selectin in AAV compared to healthy controls. In another study, levels of sE-selectin were also found to be not increased in patients with active WG (30). One explanation for the reported conflicting results regarding sE-selectin could be that induction of this adhesion molecule has to be considered as an early event in the inflammatory process. Thus expression levels might differ with respect to time point of sE-selectin determination. We observed at time of initial diagnosis a higher level of sE-selectin (53.53±38.81 ng/ml) as during course of disease (39.49±31.18 ng/ml), which supports this assumption. Also, like other authors (25), we observed a correlation between sE-selectin and C-ANCA and P-ANCA, but no significant elevation could observed in patients with AAV in comparison to healthy. The noted positive correlation between sE-selectin and P-ANCA suggests that sE-selectin could play a relevant role in P-ANCA positive vasculitis. But the small number of patients with P-ANCA vasculitis limits this speculation. In contrast, the negative correlation between sE-selectin and C-ANCA advises to a less significant role of this adhesion molecule in WG. Further investigations are required to confirm this interestingly findings in larger cohorts. Other studies showed conflict results, while some authors reports elevated levels of E-selectin in different

Table I. Pearson correlation coefficient calculation of the performed follow-up analyses in 16 patients with AAV.

	C-ANCA	P-ANCA	sE-Selectin	sVCAM-1	BLyS	CRP	Creatinine
C-ANCA	r=1.00	r=-0.591 *p*=0.016	r=-0.570 *p*=0.021	r=0.110 *p*=0.684	r=0.0691 *p*=0.799	r=0.219 *p*=0.414	r=-0.276 *p*=0.300
P-ANCA	r=-0.591 *p*=0.0160	r=1.00	r=0.601 *p*=0.014	r=0.314 *p*=0.237	r=-0.065 *p*=0.809	r=-0.248 *p*=0.354	r=0.265 *p*=0.321
sE-Selectin	**r=-0.570** ***p*=0.021**	**r=0.601** ***p*=0.014**	r=1.00	**r=0.498** ***p*=0.0497**	r=-0.216 *p*=0.4222	r=-0.185 *p*=0.4925	r=0.483 *p*=0.0581
sVCAM-1	r=-0.110 *p*=0.684	r=0.314 *p*=0.237	r=0.498 *p*=0.049	r=1.00	r=-0.339 *p*=0.199	r=-0.018 *p*=0.947	**r=0.819** ***p*=0.0001**
BLyS	r=0.069 *p*=0.7992	r=-0.065 *p*=0.809	r=-0.216 *p*=0.422	r=-0.339 *p*=0.199	r=1.00	r=0.251 *p*=0.349	r=-0.294 *p*=0.269
CRP	r=0.219 *p*=0.414	r=-0.248 *p*=0.354	r=-0.185 *p*=0.492	r=-0.018 *p*=0.947	r=0.251 *p*=0.349	r=1.00	r=0.129 *p*=0.635
creatinine	r=-0.276 *p*=0.300	r=0.265 *p*=0.321	r=0.483 *p*=0.058	**r=0.819** ***p*=0.0001**	r=-0.294 *p*=0.269	r=0.129 *p*=0.635	r=1.00

Table I represents the Pearson correlation coefficient calculation of the performed follow-up analyses in 16 patients with AAV (WG n=14, mPAN/CSS=2) from the time point of diagnosis over 5 to 7 different time points. Significant correlation were observed between sE-Selectin levels and C-ANCA as well as P-ANCA titers, between sE-Selectin and sVCAM-1, between sVCAM-1 and creatinine. Also, a significant negative correlation was observed between cANCA and pANCA.

types of vasculitis (25), contrary others could not observe a significant increase, especially in WG (30). This demonstrates that urgency exists to clarify the meaning of E-selectin in vasculitis, especially in AAV.

Interestingly, our data revealed a strong correlation between sVCAM-1 and creatinine levels in patients with AAV. Previously, an up-regulation of VCAM-1 was reported in patients with rapid progressive glomerulonephritis (RPGN) due to AAV (31). Another study confirmed the correlation between sVCAM-1 and creatinine levels in AAV showing also a decrease of sVCAM-1 in phases of clinical remission (25). These findings indicate that levels of sVCAM-1 could serve as indicator for renal involvement in AAV and may serve as indicator for recovering of renal function.

Yet another relevant result of our study was the determination of increased BLyS levels in patients with WG providing first evidence for a role of that B-cell signalling pathway. Krumbholz *et al*. (32) described similar results of elevated BLyS in patients with WG and showed declining levels in patients undergoing therapy with glucocorticoids. BLyS is relevant for the development and lifetime of B-cells increasing the number of antibody producing cells (13, 16). It was shown that an excessive expression of BLyS results in an autoimmune-like phenomenon in animal models and beyond that elevated levels of BLyS were documented in different human autoimmune diseases, like RA, SLE and pSS. Moreover, a correlation was shown between serum BLyS levels and autoantibody titres in B-cell driven autoimmune diseases such as SLE (21, 22, 33). There are further advices for an important role of B-cells in the pathological pathways in WG. The treatment with rituximab, a monoclonal anti cluster of differentiation (CD)-20 antibody, in patient with intolerance or persistence to standard treatment, showed a decreased disease activity (34). So, rituximab seems to present an effective rescue therapy. However, levels of BLyS were not significantly correlated to C-ANCA titres in our study and therefore, further analysis in larger cohorts are necessary to clarify if there exists an influence of BLyS on the B-cell compartment in WG.

In summary, the results of our study suggest that VCAM-1 is released by proteolytic cleavage of the membrane-anchored form and is significantly associated with the inflammatory endothelial process in AAV. Importantly, we observed a strong correlation between sVCAM-1 levels and renal function in AAV. The results of our study are clearly limited by the relatively small number of included patients and the retrospective design. Out of the retrospective design it was not possible to calculate the authentic disease activity by vasculitis scores. Therefore, further prospective analyses are required to clarify the significance of the described markers and to confirm the correlation with clinical manifestations and/or disease activity in AAV.

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