Neutrophil-endothelial interaction in ANCA associated vasculitis

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Chapter

4

Autoantibodies against glomerular endothelial cells in ANCA-associated systemic vasculitis

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Abstract

Objectives: The prevalence of anti-endothelial cell autoantibodies (AECA) in patients with ANCA-associated systemic vasculitis (AAV) has been reported by several groups with conflicting results ranging from 8% to 100%. Types of substrate cells used for AECA testing partially explain this variation. Endothelial cells from kidney origin have been reported to be predominant in AECA binding. Therefore, we investigated AECA prevalence using a human glomerular endothelial cell (GEnC) line compared to primary human umbilical vein endothelial cells (HUVEC) which have frequently been used for AECA detection.

Methods: Sera from 43 AAV patients (29 WG, 14 MPA) with active disease were assessed for AECA positivity using cell-based ELISA. Forty serum samples from healthy controls were tested in parallel. To evaluate endothelial activation levels, soluble intercellular cell adhesion molecule 1 (sICAM-1) and vascular cell adhesion molecule 1 (sVCAM-1) were measured with capture ELISA.

Results: AECA were detected in 4 of 29 WG patients (14%) but none of 14 MPA patients were positive for AECA using GEnC as a substrate, while AECA were positive in 10% of WG patients and 14% of MPA patients on HUVEC. No significant differences were found between AAV patients and controls in AECA test. Serum levels of sVCAM-1 and sICAM-1 in AAV patients were significantly higher than in controls. However, there were no differences between AECA positive and negative patients for both of the activation markers.

Conclusion: AECA, directed against glomerular endothelial cells, have a low prevalence in AAV patients with active disease and are not correlated with endothelial activation.

Introduction

ANCA-associated systemic vasculitides (AAV) are a group of vascular disorders consisting of Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) which share many clinical manifestations, including pulmonary vasculitis, pauci-immune focal necrotizing and/or crescentic glomerulonephritis, and presence of anti-neutrophil cytoplasmic autoantibodies (ANCAs) in the circulation.[1,2] ANCA are important diagnostic markers for AAV, and the pathogenic role of ANCA has been demonstrated by in vitro and in vivo studies.[3-5] However, the involvement of blood vessel inflammation in these diseases also evoked interest into the role of anti-endothelial autoantibodies (AECA) in the pathophysiologic process. The prevalence of AECA in AAV has been reported by several groups with conflicting data ranging from 8% to 100% of AAV patients.[6-11] In a study of Holmen et al., increased binding of AECA to endothelial cells isolated from nose, kidney and lungs, which are the most frequently involved organs in AAV, was demonstrated.[9] 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 we investigated the prevalence of AECA in ANCA-associated vasculitis using a human glomerular endothelial cell (GEnC) line in comparison with primary human umbilical vein endothelial cells (HUVEC) which are frequently used in AECA detection. Further, since AECA may induce endothelial activation,[12,13] serum levels of adhesion molecules, as markers of endothelial activation, were also analyzed in this study.

Methods

Sera of patients and healthy controls

A diagnosis of WG or MPA was established when the patients fulfilled the Chapel Hill definitions for WG or MPA.[14] Serum samples were collected at the time of diagnosis, before immunosuppressive therapy started, and stored at -80°C until AECA testing. Sera of 43 patients (29 WG/14 MPA) with active disease diagnosed between 2005 and 2007 were selected randomly and included in this study. All the 43 serum samples were tested for AECA and endothelial activation markers. The sera of 40 healthy volunteers, age and sex matched, were tested as controls. More information of patients and controls is given in Table 1.

Table 1. Characteristics of patients and controls

	AAV patients		Healthy controls
	WG	MPA	
Total number, n	29	14	40
Gender, male (%)	17 (59)	10 (71)	20 (50)
Age, median (range)	62 (34-84)	70 (40-84)	54 (45-70)
ANCA-specificity, n (%)			
PR3	24 (83)	2 (14)	-
MPO	2 (7)	12 (86)	-
Other	2 (7)	0	-
Negative	1 (3)	0	-
BVAS at diagnosis, median (range)	22 (7-33)	18 (9-27)	-
Organ involvement at diagnosis, n (%)			
ENT	29 (100)	0	-
Lung	16 (55)	8 (57)	-
Kidney	19 (66)	12 (86)	-
Skin	6 (21)	4 (29)	-
Nervous system	11 (38)	6 (43)	-

AAV: ANCA-associated systemic vasculitides, WG: Wegener's granulomatosis, MPA: microscopic polyangiitis, BVAS: BirminghamVasculitis Activity Score, ENT: ear, nose and throat.

Cell culture

CiGEnC The conditionally immortalized human glomerular endothelial cell (CiGEnC) line (provided by the Academic Renal Unit, University of Bristol, UK) was used for AECA detection. Tumor antigen transfection and characteristics of the cell line have been described.[15] CiGEnC were seeded on 96-well micro titer plates (Costar, Corning Inc., Badhoevedorp, The Netherlands) and cultured in endothelial growth medium 2–microvascular (EGM2-MV, Cambrex, Wokingham, UK), containing growth factors and supplements provided by the supplier, including human fibroblast growth factor (hFGF), human recombinant long-insulin-like growth factor-1 (R3-IGF), human epidermal growth factor (hEGF), gentamicin and amphotericin (GA-1000), hydrocortisone, heparin, ascorbic acid and 5% fetal bovine serum (FBS), at 33˚C. At the permissive temperature, the cells were allowed to proliferate for about 48 hours, until confluence. Afterwards, the plates were transferred to 37˚C where the transgene was inactivated, rendering cells non-proliferative and quiescent. At the non-permissive temperature cells were cultured for another 5 days before experiments. To investigate the influence of endothelial cell stimulation on AECA binding, some of the GEnC were

incubated with 10 ng/ml tumor necrosis factor alpha (TNF-α; R&D systems, Abingdon, UK) for 4 hours prior to AECA detection.
HUVEC The human umbilical vein endothelial cells (HUVEC), isolated as described,[16] were also used as substrate for AECA detection for comparison. Second passage HUVEC cells were cultured in gelatin pre-coated 96-well micro titer plates with RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) containing 10% FCS at 37˚C. Cells were cultured for about 2 days until confluence.

AECA detection

AECA in serum samples were measured using a cell-based enzyme-linked immunosorbent assay (ELISA) with unfixed CiGEnC and HUVEC as described previously.[17] Briefly, endothelial cell coated plates were washed with Hanks balanced salt solution (HBSS; GIBCO, Life Technologies, Breda, The Netherlands). Non-specific binding was inhibited by incubating cells with blocking buffer (HBSS/0.5% BSA) for 60 min at 37˚C. After additional washing, cells were exposed to the samples 1:20 diluted in blocking buffer. After incubation at room temperature for 1 hr, cells were washed again and incubated with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma, Zwijndrecht, The Netherlands) for 1 hr at room temperature followed by 3 washes. The substrate p-nitrophenylphosphate disodium (Sigma, Zwijndrecht, The Netherlands) was added to obtain proper color reaction. After 20 min, optical density (OD) was scanned at 405 nm. In each experiment, CD31 was measured as a positive control. Samples were run in duplicate and the absolute OD values were obtained by subtracting the mean OD readings of blank wells. IgG class of AECA were considered positive when levels exceeded the mean+1SD value of samples from healthy controls (n=40).

Evaluation of endothelial activation

As endothelial activation markers, serum levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) (R&D Systems, Abingdon, UK) and soluble intercellular adhesion molecule-1 (sICAM-1) (R&D Systems, Abingdon, UK) were measured by capture ELISA according to the manufacture's instructions. The results were expressed as the concentration calculated from a standard curve.

Statistical analysis

Values of AECA titers (OD) and concentrations of sVCAM-1 and sICAM-1 were expressed as medians and analyzed for statistical differences by the two-tailed Mann-Whitney U test. Probability values of <0.05 were considered significant.

Table2. Prevalence of AECA in AAV detected on GEnC and HUVEC

	HC (n=40)	All patients (n=43)	WG (n=29)	MPA (n=14)
AECA-prevalence n (%)				
CiGEnC				
Mean+1SD†	3 (8)	4 (9)	4 (14)	0
Mean+2SD‡	2 (5)	1 (2)	1 (3)	0
HUVEC				
Mean+1SD†	8 (20)	5 (12)	3 (10)	2 (14)
Mean+2SD‡	1 (3)	1 (2)	1 (3)	0
sICAM median (range)	111 (8-252)	214 (72-438)	214 (72-401)	208 (89-438)
sVCAM median (range)	296 (102-494)	753 (413-2138)	630 (413-1322)	956 (519-2138)

†: Values over Mean+1 standard deviation (SD) of 40 healthy controls' results were considered positive, ‡: Values over Mean+2 SD of 40 healthy controls' results were considered positive. sICAM: concentrations of soluble intercellular adhesion molecule in sera, sVCAM: concentrations of soluble vascular cell adhesion molecule in sera.

Results

AECA were detected in 4 of the 29 WG patients (14%) but not in the MPA patients when GEnC were used as substrate, while 10% of sera from WG patients and 14% from MPA patients tested positive using HUVEC-based ELISA (summarized in Table 2). Pre-stimulation of the endothelial cells with TNF-α did not show obvious influence on AECA binding (data not shown). Although a few patients were positive for AECA defined as exceeding mean+1SD value of samples from healthy controls, there was no significant difference between AAV patients and controls in AECA titer when GEnC were used as substrate and levels in patients were even lower than controls with HUVEC as substrate (Figure 1).

Serum levels of sVCAM-1 and sICAM-1 were measured to evaluate in vivo endothelial activation. AAV patients with active disease had increased serum levels of sVCAM-1 and sICAM-1 when compared with healthy controls. AECA positive patients did not show higher levels of endothelial activation markers than AECA negative patients (Figure 2). No correlation could be found between levels of sVCAM-1/sICAM-1 and AECA titers.

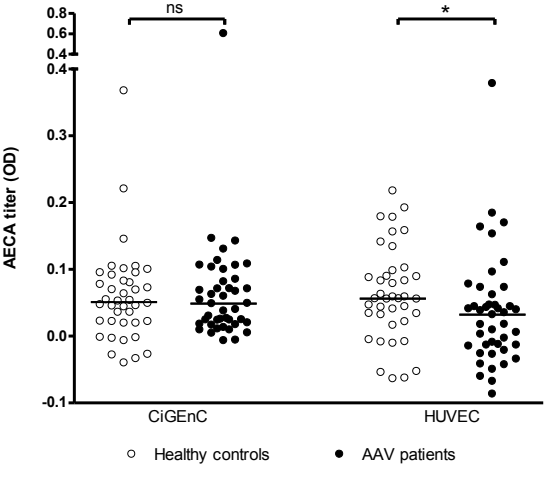

Figure 1. AECA titers (OD) in ASV patients (n=43) and healthy controls (n=40) were detected on GEnC and HUVEC. Results are expressed as absolute OD values, by subtracting readings of blank wells. Lines denote the medians which were analyzed by two-tailed Mann-Whitney U test for statistical differences.*: p<0.05, ns: not significant.

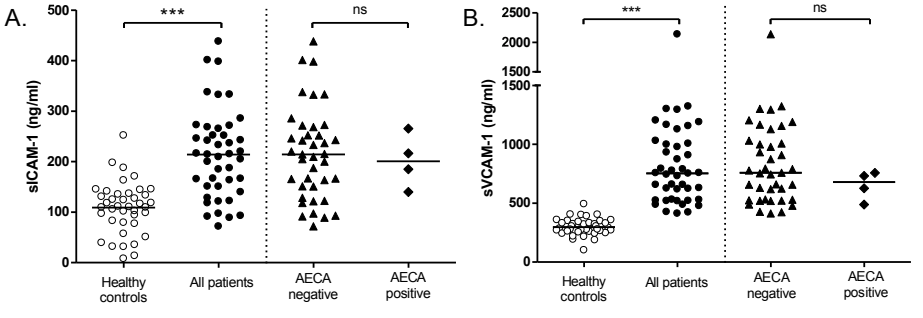

Figure 2. Elevated levels of endothelial activation markers in ASV patients compared to healthy controls. Serum levels of sICAM-1 (A) and sVCAM-1 (B) were compared between controls (n=40) and ASV patients (n=43), or between AECA negative (n=38) and AECA positive (n=4) patients. Results were expressed as the concentration (ng/ml) calculated from a standard curve. Lines denote the medians which were analyzed by two-tailed Mann-Whitney U test for statistical differences. ***: p<0.0001, ns: not significant.

Discussion

AECA have been studied for more than three decades since the first detection in human serum.[18] Diversity of techniques were used for testing AECA, such as immunofluorescence, radioimmunoassay, fluorescence-activated cell sorting, and immunoblotting.[19-22] The cell-based ELISA, which was used in our study, is a reference approach for AECA detection and has been used in most AECA screening studies recently.11 However, even using the same method, different groups reported conflicting AECA prevalence in AAV patients, which varied from 8% to 100%.[6-11] This difference could be partially explained by the use of fixation of cells. In some early studies, cultured HUVEC were fixed before being incubated with serum samples, to avoid non-specific immunoglobulin binding and loss of

cells. Fixation, on the other hand, makes cells permeable and, at least in part, AECA positivity could be due to binding to intracellular compounds. Another reason for the variation could be the difference in endothelial cells used as substrate in the AECA test. AAV is a group of diseases characterized by necrotizing damage of small-sized vessels.[1,2] Holmen et al. showed in their study that AECA binding was, to some extent, organ specific, by isolating endothelial cells from kidney, nose or lung in order to use these cells as a substrate for AECA detection. AECA bound differentially to these substrates suggesting a differential pathologic role of AECA in WG.[9,23] Therefore, in comparison with HUVEC, which is considered macrovascular EC, renal microvascular EC (such as GEnC) could be a better candidate for AECA detection in AAV. However, our data did not show a high prevalence of AECA which was reported to be 71% in Holmen's study using human kidney endothelial cells.[9] To our knowledge, no specific antigens have been demonstrated for AECA, and AECA recognize a wide rage of autoantigens such as constitutively expressed or cytokine-induced cryptic antigens, adhesion molecules and extracellular matrix components.[24] In Holmen's study, the endothelial cells that they used were isolated from whole kidney, instead of glomeruli, which may display a broader spectrum of antigens for AECA binding resulting in a higher percentage of positive results. Moreover, they used flow cytometry, other than cell-based ELISA, to measure AECA which includes different ways for cell preparation and different methods to set the cut-off value for a positive result, making the results of both methods incomparable.

The pathogenic role of AECA in AAV is controversial. It has been suggested that, in patients with WG or MPA, AECA cause EC damage through antibody-dependent cell-mediated cytotoxicity (ADCC). In the same way, ANCA could play a central role in case they recognize planted or in situ expressed proteinase 3 (PR3) or myeloperoxidase (MPO) on the EC surface, resulting in ADCC of endothelial cells.[10,25] Expression of PR3 on EC has been shown by Mayet et al., but their results have not been confirmed by other groups.[26] Ballieux et al. even denied a prominent role of ANCA mediated ADCC of EC in WG pathogenesis by showing that cultured HUVEC, pre-incubated with PR3 or MPO, followed by ANCA or rabbit anti-PR3 or anti-MPO, were not susceptible to ADCC.[27] Triggering of EC activation was otherwise considered as a pathogenic effect of AECA in AAV. Some in vitro studies showed that AECA induce production of IL-1β, IL-6, IL-8 and monocyte chemotactic factor-1, and activated EC increase leukocyte adhesion by up-regulating the expression of adhesion molecules.[12,13] Our data showed increased levels of sVCAM-1 and sICAM-1 in the circulation of AAV patients with active disease, however, levels of these adhesion molecules did not correlate with

AECA titers and there were no significant differences between AECA positive and negative patients with regard to endothelial activation markers. These results suggest that either AECA detected with the current methods are not pathogenic, or the presence of AECA is not the only factor influencing endothelial activation.

In conclusion, AECA against glomerular endothelial cells have a low prevalence in AAV patients with active disease and are not correlated with endothelial activation. It could be helpful to use more than one type of substrate cells for AECA testing in order to increase their detection rate. However, elucidating the antigens of AECA in AAV is a prerequisite for further assessing their diagnostic and pathogenetic role.

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