In vitro up-regulation of E-selectin and induction of interleukin-6 in endothelial cells by autoantibodies in Wegener’s granulomatosis and microscopic polyangiitis

A.C. Muller Kobold¹, R.T. van Wijk¹, C.F.M. Franssen², G. Molema¹,³, C.G.M. Kallenberg¹, J.W. Cohen Tervaert¹

Departments of Clinical Immunology¹ and Nephrology², University Hospital Groningen, and Department of Pharmacokinetics and Drug Delivery³, State University Groningen, The Netherlands.

Abstract

Objective

In patients with Wegener’s granulomatosis (WG) or microscopic polyangiitis (MPA) autoantibodies to myeloid granule proteins (ANCA), particularly proteinase 3 (Pr3) and myeloperoxidase (MPO), and to endothelial cells (AECA) are frequently detected. The role of these autoantibodies in the development of vascular injury is incompletely understood. Since the expression of E-selectin and the production of interleukin 6 by endothelial cells is an early step in the sequence of events leading to vascular injury, we examined the capacity of IgG fractions from patients with WG and/or MPA to activate endothelial cells to the expression of E-selectin and the production of IL-6. We related those findings to the presence of ANCA and AECA in the IgG preparations.

Methods

Human umbilical vein endothelial cells (HUVEC) were incubated with immunoglobulin (IgG) preparations from 28 patients (17 positive for anti-Pr3, 10 for anti-MPO, and one for anti-Pr3/MPO) with active vasculitis and from 10 healthy volunteers. The final IgG concentration in the activation assay was 2 mg/ml. TNFα (10 ng/ml) and LPS (10 ng/ml) were used as positive controls for HUVEC activation. The extent of HUVEC activation was assessed by the measurement of E-selectin expression by flow cytometry (after 4 hours of incubation) and the production of interleukin 6 by ELISA (after 24 hours).

Results

We found that 11 of the 28 ANCA positive IgG samples were capable of activating endothelial cells: six samples induced IL-6 production alone, one sample upregulated E-selectin expression alone, and four samples induced both IL-6 production and E-selectin upregulation. Five of 17 anti-Pr3 positive samples (one of which was also positive for AECA) and 6 of 10 anti-MPO positive samples (all simultaneously positive for AECA) induced endothelial cell activation. AECA positive samples that induced endothelial cell activation (n = 7) had higher AECA titres than samples that did not induce endothelial cell activation (n = 6).

Conclusion

Our data suggest that the activation of endothelial cells in patients with WG and MPA can be induced by circulating autoantibodies. Both ANCA and AECA can be responsible for this effect.

Key words

ANCA, AECA, IgG, endothelium, cell-activation, E-selectin, interleukin-6.
Introduction
Primary or idiopathic vasculitic syndromes form a group of inflammatory disorders characterized by inflammation and necrosis of blood vessels. Within the spectrum of vasculitides, small vessel vasculitides, in particular Wegener’s granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with anti-neutrophil cytoplasmic antibodies (ANCA). ANCA in those disorders are generally directed against proteinase 3 and myeloperoxidase (1, 2). The pathophysiology of small vessel vasculitis is not completely understood. Both leukocyte and endothelial cell activation are essential in the disease process of vasculitis (3). Upon cell activation the expression of adhesion molecules, which mediate the interaction between leukocytes and endothelial cells, is upregulated. The effects of ANCA on the process of cell activation and cell adhesion have only been partially unraveled. In vitro experiments have shown that ANCA have the potential to activate primed neutrophils to the increased expression of adhesion molecules and to the production of reactive oxygen species and the release of lytic enzymes (4-9). Furthermore, both anti-Pr3 and anti-MPO positive IgG fractions from patients with ANCA associated vasculitides have been demonstrated to activate endothelial cells in vitro. This activation results in the increased expression of adhesion molecules, such as E-selectin, VCAM-1 and ICAM-1, and the increased expression of tissue factor (10-14). In patients with WG or MPA, anti-endothelial cell antibodies (AECA) have also been demonstrated in 80% of patients (15). These AECA may also be candidates for the observed endothelial cell activation by IgG preparations from patients with WG or MPA (16).

In the present study, we investigated whether IgG preparations from patients WG or MPA are capable of stimulating the expression of E-selectin and the production of interleukin 6 (IL-6) by cultured endothelial cells. In order to analyze the differential role of various autoantibodies in endothelial cell activation, we related our results to the presence of anti-Pr3 ANCA, anti-MPO ANCA, and AECA in these IgG preparations.

Patients and methods

Introduction

Primary or idiopathic vasculitic syndromes form a group of inflammatory disorders characterized by inflammation and necrosis of blood vessels. Within the spectrum of vasculitides, small vessel vasculitides, in particular Wegener’s granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with anti-neutrophil cytoplasmic antibodies (ANCA). ANCA in those disorders are generally directed against proteinase 3 and myeloperoxidase (1, 2). The pathophysiology of small vessel vasculitis is not completely understood. Both leukocyte and endothelial cell activation are essential in the disease process of vasculitis (3). Upon cell activation the expression of adhesion molecules, which mediate the interaction between leukocytes and endothelial cells, is upregulated. The effects of ANCA on the process of cell activation and cell adhesion have only been partially unraveled. In vitro experiments have shown that ANCA have the potential to activate primed neutrophils to the increased expression of adhesion molecules and to the production of reactive oxygen species and the release of lytic enzymes (4-9). Furthermore, both anti-Pr3 and anti-MPO positive IgG fractions from patients with ANCA associated vasculitides have been demonstrated to activate endothelial cells in vitro. This activation results in the increased expression of adhesion molecules, such as E-selectin, VCAM-1 and ICAM-1, and the increased expression of tissue factor (10-14). In patients with WG or MPA, anti-endothelial cell antibodies (AECA) have also been demonstrated in 80% of patients (15). These AECA may also be candidates for the observed endothelial cell activation by IgG preparations from patients with WG or MPA (16).

In the present study, we investigated whether IgG preparations from patients WG or MPA are capable of stimulating the expression of E-selectin and the production of interleukin 6 (IL-6) by cultured endothelial cells. In order to analyze the differential role of various autoantibodies in endothelial cell activation, we related our results to the presence of anti-Pr3 ANCA, anti-MPO ANCA, and AECA in these IgG preparations.

Patients and methods

Patients and controls

The patient group consisted of consecutive patients admitted to our hospital or seen at the outpatient clinic with a diagnosis of WG or MPA. The diagnoses of WG and MPA were established according to clinical and histological criteria (2). All patients fulfilled the Chapel Hill Consensus Conference definitions for WG and/or MPA (17). Vasculitis disease activity was measured according to the Birmingham Vasculitis Activity Scoring index (BVAS) (18). Healthy laboratory personnel served as normal controls.

IgG isolation

Purified immunoglobulin G fractions were prepared from plasma samples of patients with active vasculitis, before treatment was instituted, and from healthy controls. EDTA anti-coagulated blood samples were drawn and immediately put on ice. Next, samples were centrifuged at low speed (1000 rpm 10 min.) to prevent leukocyte activation. The supernatant plasma was subsequently centrifuged at 3100 rpm for 10 min to spin down the platelets. Finally, the plasma was recalcified by the addition of a surplus of calcium.

Initially we used sequential ammonium sulphate precipitation and protein G chromatography (fast flow protein G, Pharmacia Fine Chemicals AB, Uppsala, Sweden). Since endotoxin contamination could not be excluded using this method, and since it involved many time-consuming steps, immunoglobulin preparations were also isolated using a commercially available protein G column (MabTrap, Pharmacia Biotech, Uppsala, Sweden). This method is rapid and can be carried out in a sterile hood, thus minimizing the risk of endotoxin contamination. In brief, serum samples were diluted with an equal volume of binding buffer (MabTrap, Pharmacia) and infused on a MabTrap column using a syringe. Subsequently, the column was washed with binding buffer and then eluted with elution buffer (MabTrap). The collected IgG fractions were dialysed overnight at 4°C against sterile phosphate buffered saline, pH 7.4. The column was washed thoroughly with binding buffer and stored until re-use.
Protein content of the fractions was analysed according to Bradford (BioRad Protein Assay, BioRad Laboratories GmbH, München, Germany). The fractions were tested for purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE).

All IgG preparations were tested for endotoxin contamination using the limulus amoebocyte assay (Bio-Whittaker, Boehringer Ingelheim Bioproducts Partnership, vervier, Belgium). An endotoxin concentration < 0.5 U/ml was considered negative. To rule out possible effects of endotoxin contamination, experiments yielding positive results were repeated with the addition of 50 mg/ml polymyxin B (Sigma Chemical Co, St Louis, USA).

The ANCA titre was determined prior to and after isolation in all samples. IgG preparations were centrifuged (15,000 rpm, 15 min) prior to their use in the stimulation experiments to remove aggregates.

**ANCA detection**

ANCA were detected by indirect immunofluorescence on ethanol-fixed granulocytes as previously described (19). Patient and control sera were tested at a dilution of 1:20, and further at two-fold dilutions. Slides were read by two independent observers, and a titre 1:≥40 was considered positive.

The specificity of ANCA for either Pr3, MPO or human leukocyte elastase (HLE) was detected by direct ELISA as previously described (20). Sera were considered positive for one of the aforementioned specificities when values exceeded the mean + 2 standard deviations of normal controls (n = 50).

**Endothelial cell isolation and culture**

In all experiments human umbilical vein endothelial cells (HUVEC) were used. HUVEC were isolated as previously described (21) with minor modifications (22). Briefly, endothelial cells were harvested from human umbilical cord veins using chymotrypsin (Sigma Chemical Co) and cultured in 1% gelatin- (Sigma Chemical Co) coated 25 cm² tissue culture flasks (Costar, Cambridge, USA) in endothelial cell growth medium (RPMI 1640 (Bio-Whittaker), 20% fetal calf serum (Integro BV, Zaandam, The Netherlands), endothelial cell growth factor 50 mg/ml, heparin 5 U/ml (Leo Pharmaceuticals Products BV, Weep, The Netherlands), L-glutamine 2 mM (Gibco-BRL, Paisley, Scotland), penicillin 100 U/ml (Yamanouchi Pharma BV, Leiderdorp, The Netherlands) and 0.1 mg/ml streptomycin (Radiumfarma, Fisiopharma, Italy). Cells were characterized by microscopic analysis, in which a typical endothelial cobblestone morphology was identified, and by the demonstration of the presence of von Willebrand factor and CD31. HUVEC cells were used at passages three to five.

**Anti-endothelial cell antibody (AECA) assay**

Anti-endothelial cell antibodies were detected in serum using a cyto-ELISA on unfixed endothelial cells (15, 16). In brief, endothelial cells were isolated and cultured as described above until confluence and used at passages 3 to 5. The cells were washed and incubated for 45 min at 4°C with a washing buffer consisting of RPMI (Bio-Whittaker) pH 6, supplemented with 1% fetal calf serum (Integro BV). Subsequently, serum samples were incubated in duplicate at three-fold dilutions starting with a dilution of 1:30 for 90 minutes at 4°C. Cells were then gently washed 3 times with washing buffer and alkaline phosphatase conjugated goat anti-human IgG antibody (Jackson Immunoresearch Laboratories Inc., Westgrove, PA, USA) was incubated at a dilution of 1:3000 for 1 hr at 4°C. After washing, the bound antibody was detected using p-nitrophenyl-phosphate disodium as a substrate. The optical densities (OD) were read at 405 nm. In all tests a positive reference serum was used at two-fold dilutions, starting at a dilution of 1:50 to obtain a calibration curve. The OD value of the 1:100 dilution of this reference sample was set at 1 arbitrary unit. Sera were expressed in arbitrary units and corrected for the dilution factor used. Sera were considered positive for AECA when values exceeded the mean + 2 SD of normal controls (n = 17).

**Endothelial cell stimulation by IgG preparations**

To investigate the effects of the IgG preparations on endothelial cell activation, endothelial cells were incubated with IgG preparations at a final concentration of 2 mg/ml. As positive controls tumor necrosis factor α (TNFα) (Boehringer Ingelheim, Ingelheim am Rhein, Germany) at a concentration of 10 ng/ml and/or endotoxin (Sigma Chemical Co.) at a concentration of 10 ng/ml were used.

**Analysis of endothelial cell activation**

Endothelial cell activation was analysed in two ways, in terms of E-selectin expression as assessed by flow cytometry, and interleukin-6 production measured by ELISA.

IgG preparations were considered to induce endothelial cell activation, as demonstrated by an increased E-selectin expression or IL-6 production, when values of E-selectin expression or IL-6 production were above the cut-off point, defined as the mean value of the healthy control samples (10 healthy control samples per experiment), which were run in the same experiment, plus two times their standard deviation.

**Analysis of E-selectin expression by flow cytometry**

After stimulating the endothelial cells for 4 hours to induce E-selectin expression, the cells were detached from the surface by trypsin/EDTA (Sigma Aldrich Co Ltd, Irvine, UK), at 37°C, and washed with 20% fetal calf serum (Integro) in RPMI (Bio-Whittaker). The first antibody (anti-E-selectin, H187-ABC, which was kindly provided by Prof. M.A. Gimbrone Jr., Boston, MA, USA) was incubated for 30 minutes at 4°C. After washing the cells with RPMI supplemented with 5% fetal calf serum, a FITC conjugated goat anti-mouse antibody (Dako, Dakopatts, Glostrup, Denmark) was added for 30 minutes at 4°C. Subsequently, the cells were washed and stored until flow cytometric analysis was performed. Analysis of surface marker expression was performed using a Coulter Epics ELITE flow cytometer (Coulter, Hialeah, Florida, USA) on the same day. Data were expressed as mean fluorescence intensities (MFI).

**Analysis of interleukin 6 production by ELISA**

IL-6 levels were determined in the supernatant of endothelial cells which were...
incubated with IgG fractions for 24 hrs. IL-6 concentrations were analyzed by ELISA according to Helle et al. (23) with modifications. In brief, microtitre plates (no. 9018, Costar, The Netherlands) were coated overnight at room temperature (RT) in PBS with a monoclonal antibody against human IL-6 (CLB.MIL6/16, Central Laboratory of Blood Transfusion Services (CLB), Amsterdam, The Netherlands) at a dilution of 1:500. After washing with 0.025 M Tris.HCl, 0.15M NaCl and 0.05% Tween-20, the plates were blocked with 2% BSA/0.05% Tween-20 in PBS for 1 hour at RT. As a reference, recombinant IL-6 was used (highest concentration 2000 pg/ml).

Subsequently, patient and healthy control IgG samples (two-fold dilutions) were incubated for 2 hours. Wells were washed and biotinylated sheep polyclonal antibody against human IL-6 was added (CLB.SIL6-D, CLB, Amsterdam) at a dilution of 1:3000 for 1 hour at RT in the dark. IL-6 was detected by the addition of streptavidin horse radish peroxidase (CLB, M2032 STREPTA-E+) for 30 min at RT in the dark. Finally, the plates were washed and the color reaction was initiated by the addition of 100 µl per well of 2N H2SO4. The plates were scanned at 450 - 575 nm.

IgG preparations were considered to induce endothelial cell activation, as demonstrated by IL-6 production, when the values for the IL-6 concentrations were above the cut-off point, defined as the mean value of the healthy control samples run in the same experiment + 2 SD.

Table I. Patient and IgG characteristics.

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Renal involvement</th>
<th>Ser. creatinine (µmol/L)</th>
<th>CRP (mg/L)</th>
<th>Birmingham VAS</th>
<th>ANCA specificity</th>
<th>ANCA before</th>
<th>ANCA after</th>
<th>AECA (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>26</td>
<td>WG</td>
<td>yes</td>
<td>120</td>
<td>22</td>
<td>21</td>
<td>MPO</td>
<td>640</td>
<td>160</td>
<td>33.1</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>74</td>
<td>WG</td>
<td>yes</td>
<td>289</td>
<td>191</td>
<td>45</td>
<td>MPO, Pr3</td>
<td>40</td>
<td>160</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>22</td>
<td>WG</td>
<td>no</td>
<td>76</td>
<td>4</td>
<td>7</td>
<td>Pr3</td>
<td>640</td>
<td>80</td>
<td>21.5</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>40</td>
<td>WG</td>
<td>no</td>
<td>76</td>
<td>4</td>
<td>6</td>
<td>Pr3</td>
<td>160</td>
<td>160</td>
<td>12.8</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>67</td>
<td>WG</td>
<td>no</td>
<td>95</td>
<td>4</td>
<td>12</td>
<td>Pr3</td>
<td>80</td>
<td>40</td>
<td>6.5</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>60</td>
<td>WG</td>
<td>yes</td>
<td>268</td>
<td>31</td>
<td>29</td>
<td>Pr3</td>
<td>640</td>
<td>80</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>46</td>
<td>WG</td>
<td>yes</td>
<td>110</td>
<td>53</td>
<td>13</td>
<td>Pr3</td>
<td>640</td>
<td>160</td>
<td>8.2</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>80</td>
<td>WG</td>
<td>yes</td>
<td>136</td>
<td>163</td>
<td>21</td>
<td>Pr3</td>
<td>&gt; 640</td>
<td>ND</td>
<td>17.2</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>77</td>
<td>WG</td>
<td>yes</td>
<td>874</td>
<td>101</td>
<td>32</td>
<td>MPO</td>
<td>&gt; 640</td>
<td>&gt; 640</td>
<td>30.8</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>52</td>
<td>MPA</td>
<td>yes</td>
<td>455</td>
<td>29</td>
<td>21</td>
<td>Pr3</td>
<td>40 - 80</td>
<td>40</td>
<td>35.1</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>52</td>
<td>WG</td>
<td>no</td>
<td>74</td>
<td>111</td>
<td>9</td>
<td>Pr3</td>
<td>160</td>
<td>80</td>
<td>29.3</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>16</td>
<td>MPA</td>
<td>yes</td>
<td>52</td>
<td>76</td>
<td>12</td>
<td>MPO</td>
<td>640</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>67</td>
<td>WG</td>
<td>yes</td>
<td>110</td>
<td>192</td>
<td>3</td>
<td>Pr3</td>
<td>40</td>
<td>40</td>
<td>1.8</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>67</td>
<td>WG</td>
<td>yes</td>
<td>211</td>
<td>71</td>
<td>33</td>
<td>Pr3</td>
<td>320</td>
<td>320</td>
<td>20.6</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>66</td>
<td>WG</td>
<td>yes</td>
<td>780</td>
<td>40</td>
<td>20</td>
<td>Pr3</td>
<td>320</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>57</td>
<td>MPA</td>
<td>yes</td>
<td>634</td>
<td>105</td>
<td>12</td>
<td>MPO</td>
<td>640</td>
<td>80</td>
<td>30.6</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>62</td>
<td>WG</td>
<td>yes</td>
<td>659</td>
<td>151</td>
<td>26</td>
<td>Pr3</td>
<td>&gt; 640</td>
<td>ND</td>
<td>7.9</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>51</td>
<td>MPA</td>
<td>yes</td>
<td>233</td>
<td>96</td>
<td>19</td>
<td>MPO</td>
<td>&gt; 640</td>
<td>160</td>
<td>36.9</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>53</td>
<td>WG</td>
<td>yes</td>
<td>97</td>
<td>20</td>
<td>21</td>
<td>Pr3</td>
<td>640</td>
<td>80</td>
<td>23.1</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>71</td>
<td>WG</td>
<td>yes</td>
<td>249</td>
<td>122</td>
<td>30</td>
<td>Pr3</td>
<td>&gt; 640</td>
<td>320</td>
<td>10.9</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>70</td>
<td>MPA</td>
<td>yes</td>
<td>482</td>
<td>53</td>
<td>21</td>
<td>MPO</td>
<td>320</td>
<td>ND</td>
<td>20.2</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>91</td>
<td>WG</td>
<td>yes</td>
<td>1013</td>
<td>99</td>
<td>23</td>
<td>Pr3</td>
<td>160</td>
<td>40</td>
<td>18.1</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>81</td>
<td>WG</td>
<td>yes</td>
<td>479</td>
<td>5</td>
<td>16</td>
<td>MPO</td>
<td>320</td>
<td>320</td>
<td>37.3</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>71</td>
<td>WG</td>
<td>yes</td>
<td>211</td>
<td>34</td>
<td>20</td>
<td>Pr3</td>
<td>320</td>
<td>80</td>
<td>14.4</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>77</td>
<td>WG</td>
<td>yes</td>
<td>316</td>
<td>5</td>
<td>21</td>
<td>MPO</td>
<td>640</td>
<td>160</td>
<td>29.9</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>75</td>
<td>WG</td>
<td>yes</td>
<td>190</td>
<td>18</td>
<td>18</td>
<td>MPO</td>
<td>&gt; 640</td>
<td>40</td>
<td>51</td>
</tr>
<tr>
<td>27</td>
<td>F</td>
<td>37</td>
<td>WG</td>
<td>yes</td>
<td>829</td>
<td>262</td>
<td>23</td>
<td>MPO</td>
<td>320</td>
<td>20</td>
<td>58.4</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>31</td>
<td>WG</td>
<td>yes</td>
<td>93</td>
<td>190</td>
<td>21</td>
<td>Pr3</td>
<td>&gt; 640</td>
<td>80</td>
<td>45.8</td>
</tr>
</tbody>
</table>

WG: Wegener’s granulomatosis; MPA: Microscopic polyangiitis; Pr3, proteinase 3; MPO, Myeloperoxidase; AECA, anti-endothelial cell antibodies (cut-off point 27 units).
10 for anti-MPO antibodies, whereas one patient was simultaneously positive for anti-Pr3 and anti-MPO antibodies. Healthy laboratory personnel served as the healthy controls. The patients’ characteristics are shown in Table I.

**AECA**

Thirteen of 28 patients (46%) were positive for AECA. Of these 13 patients, 4 had anti-Pr3 antibodies (24% of all anti-Pr3 positive patients), 8 had anti-MPO antibodies (80% of all anti-MPO positive patients), and 1 had both anti-Pr3 and anti-MPO antibodies. Data are presented in Table I and Figure 1. ANCA in the AECA positive samples were more frequently directed against MPO than against Pr3 \( (p = 0.0069) \). No correlations were found between the AECA and ANCA titres, nor between the AECA titre and disease activity, as determined by the BVAS score, CRP values or serum creatinine. In addition, no relationship was found between the number of organs involved and the presence of AECA.

**Endothelial cell activation (E-selectin expression and IL-6 production)**

Upon stimulation with 10 ng/ml TNF\( \alpha \) or 10 ng/ml endotoxin, E-selectin expression on endothelial cells increased compared to basal expression. In addition, stimulation with TNF\( \alpha \) or endotoxin resulted in high IL-6 production, whereas only low levels of IL-6 could be detected without stimulus. Polymyxin B alone or in combination with TNF\( \alpha \) had no effect on HUVEC activation, whereas endothelial cell activation with 10 \( \mu \)g/ml of LPS was inhibited by the addition of 50 \( \mu \)g/ml of polymyxin B (Fig. 2). E-selectin expression was induced by IgG samples from 5 patients (2 anti-Pr3 and 3 anti-MPO). Four of these 5 patients were also positive for AECA (1 anti-Pr3, 3 anti-MPO). No differences in the E-selectin inducing capacity of IgG samples were found between patients with WG or MPA, between Pr3 ANCA- or MPO ANCA-containing samples, between AECA positive or AECA negative samples, or between samples contaminated with endotoxin (and tested in combination with polymyxin B) and samples without endotoxin.

Production of IL-6 by endothelial cells was induced by IgG samples from ten patients (4 anti-Pr3, 6 anti-MPO). Seven of these 10 patients were also positive for AECA (1 anti-Pr3, 6 anti-MPO). ANCA in AECA positive samples that
induced IL-6 production by endothelial cells were directed against MPO more frequently than against Pr3 (p = 0.0333). No differences in the IL-6 inducing capacity of IgG samples were found between patients with WG or MPA, between AECA positive or AECA negative samples, or between samples contaminated with endotoxin (and tested in combination with polymyxin B) and samples without endotoxin.

Taken together, within the group of patients with WG or MPA, 11 of 28 patient IgG fractions induced endothelial cell activation, either by inducing E-selectin expression alone, or by inducing IL-6 production alone, or by inducing both.

7/11 ANCA positive IgG preparations that induced endothelial cell activation were also positive for AECA and 4 of these were AECA negative. Otherwise, 7 of 13 AECA positive IgG samples (6 anti-MPO, 1 anti Pr3) were capable of inducing endothelial cell activation. A schematic representation of these results is given in Figure 3. The results of representative experiments on the induction of E-selectin expression and IL-6 production are shown in Figure 4.

**Discussion**

In this study we found that a significant number of IgG fractions from patients with WG or MPA were capable of activating endothelial cells *in vitro*. We related our findings to the presence of ANCA and AECA, which are both candidates for the activation of endothelial cells (10-16), and found that both AECA positive/ANCA positive and AECA negative/ANCA positive samples could induce endothelial cell activation.

Some controversy exists as to whether the capacity of ANCA positive IgG to activate endothelial cells is due to the presence of ANCA IgG in these samples (10, 11, 13, 14, 24) or rather to the presence of other autoantibodies such as AECA (12, 16). Both del Papa et al. (16) and Johnson et al. (12) demonstrated that the capacity of ANCA positive IgG to activate endothelial cells did not disappear when anti-Pr3 reactivity was removed by pre-absorbing the IgG fractions on a Pr3 column or by co-incubating the IgG fractions with purified ANCA antigens, respectively.

We found that 11 of 28 ANCA positive IgG fractions were able to induce endothelial cell activation, resulting in E-selectin expression and/or IL-6 production. Within this group some samples were ANCA positive but AECA negative, whereas others were positive for both ANCA and AECA. Interestingly, these ANCA positive, AECA negative samples all contained anti-Pr3 antibodies. Mayet et al. showed that the activation of endothelial cells *in vitro* by cytokines results in the translocation of Pr3 from the cytoplasm to the cell membrane (13,
25). Consequently, Pr3 may be accessible for anti-Pr3 antibodies. However, at least two groups were unable to confirm the expression of Pr3 on endothelial cells (26, 27). Alternatively, Pr3 may bind to endothelial cells as a planted antigen, and thus serve as a target for ANCA. In vitro studies have demonstrated that Pr3 is capable of binding to endothelial cells (12, 28) or the extracellular matrix (29).

Since a specific receptor for Pr3 may be present on endothelial cells (30), the activation of endothelial cells may be induced by Pr3/ANCA complexes which could have been present in the IgG preparations that we used.

Endothelial cell activation might also be induced by anti-MPO positive samples. In the present study, we found that all of the anti-MPO positive IgG fractions that induced endothelial cell activation were simultaneously positive for AECA. Therefore, AECA and not anti-MPO may have been responsible for the endothelial cell activation by these IgG fractions. Indeed, MPO expression on endothelial cells has not been demonstrated (27) and no specific MPO receptor has been found on endothelial cells. Furthermore, moderately higher AECA titres were observed in samples that activated endothelial cells than in AECA positive samples that did not activate endothelial cells. This relationship between AECA titre and endothelial cell activation may suggest a pathophysiological role for AECA in WG or MPA. Unfortunately, the target antigens of AECA in these diseases are not yet known (15), a fact which precludes for the moment any further elucidation of the mechanisms involved.

Surprisingly, patients with IgG fractions that stimulated endothelial cell activation were not suffering from a more severe disease pattern than those with fractions that did not stimulate endothelial cell activation. Therefore, the clinical significance of these in vitro findings is not yet clear.

IgG fractions commonly harbour constituents other than IgG, such as cytokines and natural autoantibodies, including autoantibodies to cytokines, which may carry cytokines into the fractions because of their high affinity for these proteins. The IgG preparations used in this study were isolated on a protein G column, and for this reason naturally occurring autoantibodies may have been isolated as well. However, the stimulation experiments were performed with both patient and healthy control IgG, both of which were isolated over a protein G column and thus both possibly containing naturally occurring autoantibodies. By comparing the results for patient IgG and healthy control IgG, both of which were isolated over a protein G column and thus both possibly containing naturally occurring autoantibodies, we probably excluded the effects of these natural antibodies in these experiments.

In conclusion, IgG from patients with WG or MPA are capable of stimulating endothelial cells, as assessed by the induction of either E-selectin expression or IL-6 production. Both samples posi-
Endothelial cell activation induced by ANCA? A.C. Muller Kobold et al.

tive for AECA and samples negative for AECA were capable of inducing endothelial cell activation. Importantly, endothelial cell activation by AECA-negative IgG samples was only observed in anti-Pr3 positive samples, suggesting that Pr3 is expressed on endothelial cells. The lack of a relationship between endothelial cell activating capacity and disease severity does not support a direct pathophysiological role for these in vitro findings.

Acknowledgements

The authors wish to thank H. I. Bakker for her technical assistance, Dr. C.A. Stegeman for the inclusion of patients and Dr. P.C. Limburg for his valuable advice.

References