Anti–Endothelial Cell Autoantibodies Selectively Activate SAPK/JNK Signalling in Wegener’s Granulomatosis

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ABSTRACT

The pathogenic role of anti–endothelial cell antibodies (AECA) in vascular injury is debated. It was previously shown that many patients with Wegener’s granulomatosis (WG) have AECA that react with human kidney microvascular endothelial cells (EC). In addition, during active disease, renal endothelium strongly expresses the inflammatory molecules vascular adhesion protein-1 (VAP-1) and MHC class I–related antigen A (MICA). This study sought to determine whether AECA mediates this upregulation of VAP-1 and MICA and to define better the signaling pathways that are activated by these autoantibodies upon binding to EC in the kidney. Stimulation of human kidney microvascular EC with AECA IgG upregulated surface expression of MICA and VAP-1, elicited a rapid Ca2+ flux, induced high levels of the chemokines monocyte chemoattractant protein-1 and granulocyte chemotactic protein-2, induced specific phosphorylation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) and the transcription factors c-Jun and activating transcription factor-2, and activated NF-κB. Specific inhibitors of SAPK/JNK significantly reduced AECA-induced chemokine production and phosphorylation of c-Jun and activating transcription factor-2 and abrogated protein expression of MICA but not VAP-1. In kidney sections from patients with WG, infiltrating cells that expressed the ligand for MICA (NKG2D) were identified, as were CD8+ and 32+ T cells. In conclusion, AECA may be involved in the pathogenesis of WG, and the SAPK/JNK pathway and the endothelial inflammatory protein VAP-1 may be novel therapeutic targets for vasculitis.


Received November 28, 2006. Accepted June 7, 2007. Published online ahead of print. Publication date available at www.jasn.org.

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Wegener’s granulomatosis (WG) is characterized as a necrotizing granulomatous inflammation and vasculitis of small- to medium-sized vessels mainly in the upper airways, the lower respiratory tract, and kidneys. There is kidney involvement in the majority (85%) of patients with WG.1 The positive laboratory finding of cytoplasmic antineutrophil cytoplasmic antibodies (ANCA) directed to proteinase 3 (PR3) has been helpful in the diagnosis, with specificity of approximately 95% in active WG. A probable pathogenic role for ANCA in WG has been proposed by various in vitro studies.2–4 Another population of antibodies, anti–endothelial cell antibodies (AECA), have been impli-
icated in the pathogenesis of various vasculitides, including WG, where a high prevalence in sera has been identified. The support that AECA might be pathogenic is sustained by recognition of endothelial surface proteins, induction of various adhesion molecules on endothelial cells (EC) as well as of secretion of cytokines and chemokines; promotion of thrombotic events by increased production of tissue factor and von Willebrand factor, and fluctuation with the clinical activity of the disease. Experimental in vitro and in vivo models support a potential pathogenic role for AECA in sustaining immune-mediated vessel inflammation.

Stress-activated protein kinases (SAPK), which are members of the mitogen-activated protein kinase (MAPK) family, include c-Jun N-terminal kinase (JNK) and p38 MAPK. JNK and p38 MAPK are activated by various stimuli, including stress, ultraviolet irradiation, and proinflammatory cytokines. Studies have shown that ANCA antibodies lead to activation of p38 MAPK. However, the role of stress-activated pathways in AECA–induced pathogenesis has not been elucidated.

In a previous report, we demonstrated that AECA in patients with WG showed increased binding to EC isolated from the nose, kidney, and lungs, which are the main organs affected in WG, as compared with unrelated human umbilical vein EC (HUVEC) and liver EC. In another recent study, we demonstrated a strong expression of two inflammatory molecules, vascular adhesion protein-1 (VAP-1) and MHC class I–related antigen A (MICA), on kidney endothelium of patients with WG during active disease. VAP-1 is an endothelial molecule that possesses both adhesive (mediates leukocyte trafficking to sites of inflammation) and enzymatic properties in vitro. MICA, known to be induced by external stress, is the ligand for NKG2D receptors found on many cells within the immune system, and it is well documented that ligand engagement of NKG2D results in cell–mediated cytotoxicity.

These observations prompted us to investigate whether IgG AECA from patients with WG would activate and mediate upregulation of VAP-1 and MICA on human kidney microvascular EC (HKMEC). Furthermore, in an experimental animal model, AECA isolated from patients with WG were capable of inducing vasculitis–like lesions after immunization. However, the mechanisms and signaling pathways used by these antibodies in causing kidney vascular damage is not known. We therefore also followed the signal transduction pathway used by AECA to activate HKMEC. So far, most of the work involving AECA has been investigated using clinically irrelevant EC targets such as HUVEC. In this study, we used one of the relevant EC targets in necrotizing vasculitis—HKMEC—thereby underscoring the importance of our study.

RESULTS

Characterization of HKMEC

With the use of flow cytometry, phenotypic characterization of HKMEC showed that the cells expressed all of the endothelial markers tested (Table 1). In addition, light microscopy demonstrated endothelial morphology and maintained contact inhibition for approximately eight passages, after which the cells ceased to divide. We used freshly isolated HKMEC for all experiments involving VAP-1, whereas HKMEC in passages three to four (Figure 1A) were used for the remaining studies. In matrigel, these cells formed capillary-like structures, indicating their endothelial origin (Figure 1B), and their glomerular origin was confirmed by the presence of fenestræ using scanning electron microscopy (Figure 1C).

PR3-Depleted IgG from Patients with WG Showed Binding Capacity and Induced Rapid Calcium Flux in HKMEC

The flow-through from the PR3 column did not show any PR3 activity (<10 U). The IgG concentration after anti-PR3 absorption and fast protein liquid chromatography was estimated to a total of 20 mg by the Mancini method. Anti-PR3–depleted WG IgG was tested for their sustained capacity to bind HKMEC (Figure 1D) and was found to bind strongly to HKMEC.

WG IgG Did Not Bind HUVEC

No binding of PR3-depleted IgG AECA to primary human renal epithelial cells or polymorphonuclear monocytes (PMN) was found by flow cytometry (Figure 1D), demonstrating that WG IgG are not directed against other target cells. Upon PR3-depleted WG IgG stimulation, HKMEC elicited a dosage-dependent Ca2+ response that peaked after 5 to 10 s, lasted for nearly 40 s, and resulted in a Ca2+ change of 115 nM (calculated from baseline value before stimuli challenge). No Ca2+ change was detected upon stimulation with normal IgG (Figure 1E). Addition of normal or PR3-depleted IgG to HUVEC did not elicit any significant Ca2+ response as compared with HKMEC (P < 0.001).

Chemokine Levels in Unstimulated and PR3-Depleted IgG-Stimulated HKMEC

Incubation of HKMEC with WG IgG AECA did not result in a significant change in chemokine levels measured by enzyme-linked immunosorbent assay (data not shown). However, we did verify the expression of two inflammatory molecules, VAP-1, and MICA in HKMEC using specific antibodies (Figure 1F).

Table 1. Phenotypic characteristics of EC isolated from human umbilical cord vein and kidney

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>HUVEC</th>
<th>HKMEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD141</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD142b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD144</td>
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<td>+</td>
</tr>
<tr>
<td>CD105</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>α-Actin</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*a*VWF, von Willebrand factor.

bExpressed only activated EC.
significant change in the levels of growth-regulated onco-
gene-α (GRO-α) and IL-8 as compared with controls. How-
ever, the levels of monocyte chemoattractant protein 1
(MCP-1) and granulocyte chemotactic protein-2 (GCP-2)
were significantly increased as compared with the controls
(P < 0.001; Table 2). Production of MCP-1 and GCP-2 by
HKMEC was found to be PR3-depleted WG IgG dosage-de-
pendent (Table 3).

Induced Expression of MICA and VAP-1 in HKMEC
after WG PR3-Depleted IgG AECA Stimulation
Unstimulated and normal IgG-stimulated HKMEC did not

Table 2. Chemokine levels in supernatants of unstimulated, normal IgG or WG IgG-stimulated HKMEC

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>MCP-1 (pg/ml)</th>
<th>GCP-2 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>GRO-α (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<tr>
<td>Only medium</td>
<td>293 ± 155b</td>
<td>&lt;30c</td>
<td>1904 ± 609</td>
<td>915 ± 152</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>+JNK inhibitor</td>
<td>300 ± 111</td>
<td>&lt;30</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>+JNK peptide inhibitor</td>
<td>310 ± 35</td>
<td>&lt;30</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Normal IgG</td>
<td>133 ± 14b</td>
<td>216 ± 71b</td>
<td>1199 ± 61</td>
<td>670 ± 509</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+JNK inhibitor</td>
<td>140 ± 35</td>
<td>230 ± 22</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+JNK peptide inhibitor</td>
<td>143 ± 10</td>
<td>220 ± 15</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WG IgG</td>
<td>1378 ± 54b</td>
<td>1185 ± 12c</td>
<td>1699 ± 109</td>
<td>607 ± 641</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>+JNK inhibitor</td>
<td>355 ± 70c</td>
<td>44 ± 5c</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+JNK peptide inhibitor</td>
<td>338 ± 24c</td>
<td>36 ± 6c</td>
<td>NT</td>
<td>NT</td>
<td>ND</td>
<td>ND</td>
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aND, note detectable; NT, not tested.
bP < 0.01.
cP < 0.001.
Table 3. Chemokine levels secreted by HKMEC in response to various concentrations of normal or PR3-depleted WG IgG

<table>
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<th>Parameter</th>
<th>IgG Concentrations (mg/ml)</th>
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<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Normal IgG</td>
<td></td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>147 ± 25</td>
</tr>
<tr>
<td>GCP-2 (pg/ml)</td>
<td>184 ± 51</td>
</tr>
<tr>
<td>PR3-depleted WG IgG</td>
<td></td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>1329 ± 72</td>
</tr>
<tr>
<td>GCP-2 (pg/ml)</td>
<td>1007 ± 30</td>
</tr>
</tbody>
</table>

Figure 2. (A and B) Immunocytochemical analysis demonstrated that unstimulated and normal IgG-stimulated HKMEC showed no expression of MICA. (C) Stimulation with PR3-depleted WG IgG induced an intense expression of MICA (red/brown staining). (D through F) Similar results were obtained for VAP-1. (H and K) Transfected VAP-1 and MICA cells stained with VAP-1 and MICA antibodies served as positive controls (red/brown staining) (I and L) Their respective mock cells served as negative controls. (G and J) Control with secondary antibodies alone. (J through L) Cells were cytospinned before staining. (M) Immunoprecipitation of biotinylated kidney EC proteins with human normal and WG IgG showed the following: Lane 1, normal IgG (one band; 190 to 200 kD); lane 2, WG IgG (three bands; 190 to 200, 70 to 73, and 50 to 53 kD). Magnification, ×40.
stain positively for MICA (Figure 2, A and B), but incubation of HKMEC with PR3-depleted IgG resulted in intense positive staining for MICA (Figure 2C). Similar results were obtained for VAP-1 using freshly isolated HKMEC (Figure 2, D through F). The positive controls VAP-1 and MICA transfecants stained positive with anti–VAP-1 and anti-MICA antibodies (Figure 2, H and K), whereas the mock cells did not (Figure 2, I and L). Controls with secondary antibody only are shown in Figure 2, G and J. Importantly, WG IgG induced expression of VAP-1 on only freshly isolated but not cultured or subpassaged HKMEC. Conversely, MICA expression by WG IgG was induced on both freshly isolated and subcultured HKMEC (data not shown).

**PR3-Depleted IgG AECA Bind Three HKMEC Antigens**

For detection of the WG IgG autoantigen reactivity, WG IgG was immunoprecipitated with biotinylated cell surface proteins expressed on kidney EC. Figure 2M shows a representative result of the immunoblotting. WG IgG immunoprecipitated three protein bands with molecular weights of approximately 190 to 200, 70 to 73, and 50 to 53 kD (lane 2). With normal IgG, only one faint band (190 to 200 kD) was demonstrated (lane 1).

**Activation of SAPK/JNK by PR3-Depleted WG AECA IgG**

In this study, we investigated whether PR3-depleted WG AECA IgG could cause activation of various MAPK. As shown in Figure 3, we found that phosphorylated JNK MAPK was phosphorylated beginning at approximately 15 min after stimulation with WG AECA IgG. In addition, phosphorylation of the transcription factors c-Jun and activating transcription factor-2 (ATF-2) and activation of NF-κB was observed. However, there was no increase in the phosphorylation levels of p38 phosphorylated ELK-1 (pELK-1), and p44/42.

**Effects of Inhibition of JNK on AECA-Induced Activation of HKMEC**

We next investigated the role of the activation of JNK on HKMEC by using specific inhibitors for JNK1/2: SP600125 and JNK peptide inhibitor 1. We chose to use c-Jun and ATF-2, which are downstream substrates of the JNK1 and 2 pathway.8 In addition, we investigated the effects of the two JNK inhibitors on the cell surface protein expression of MICA and VAP-1 induced by WG AECA on HKMEC. We found that there was a significant increase in the phosphorylation status of c-Jun and ATF-2 after AECA WG IgG stimulation (Figure 3). The JNK inhibitor SP600125 reduced AECA-induced phosphorylation of c-Jun by 70% and ATF-2 by up to 50% at a concentration of 50 μM (Figure 4, A and B). To confirm that activation of JNK after AECA-stimulation is responsible for c-Jun and ATF-2 activation, we used a peptide inhibitor of JNK (JNK inhibitor 1) and found that it was also able to reduce AECA-induced c-Jun and ATF-2 phosphorylation (Figure 4, C and D).

Using FACS analysis, we confirmed that stimulation with WG AECA IgG led to a marked increase in protein expression levels of MICA on HKMEC. However, the expression of MICA was almost completely abrogated upon treatment with JNK MAPK inhibitor (Figure 5). MICA expression levels in HKMEC decreased to that of the negative control when treated with 50 μM JNK MAPK inhibitor. We further confirmed that WG AECA markedly increased surface expression of VAP-1 on HKMEC. However, the surface expression of VAP-1 was not significantly inhibited by either of the two JNK inhibitors (Figure 6). Furthermore, both the JNK inhibitors significantly decreased the production of the two chemokines MCP-1 and GCP-2 (P < 0.001; Table 4).

**Expression of MICA and VAP-1 in Kidney Biopsies of Patients with WG**

In kidney biopsy sections from patients with active WG disease (n = 6), we found strong expression of MICA and VAP-1, confirming our previous results13 (Figure 7, A and B). We previously reported13,15 that VAP-1 but not MICA is expressed in peritubular capillaries in normal kidneys. However, we found that MICA was strongly expressed in glomerular vessels and in epithelial cells, whereas VAP-1 was strongly expressed in the peritubular and tubular capillaries and around glomerular vessel areas in patients with active disease. Kidney glomerular endothelium stained positively for both markers.

Immunohistochemical staining for infiltrating cells in WG kidney sections showed positive staining for infiltrating cells expressing the MICA ligand NKG2D as well as cytotoxic CD8+ and γδ T cells around tubular and glomerular capillaries (Figure 7, D through F), which was negative in kidney sections.
of normal controls (Figure 7, G through I). Control staining with only secondary antibody is shown in Figure 7C.

DISCUSSION

This study provides evidence that AECA IgG in patients with necrotizing vasculitis activates and induces proinflammatory signals in EC. These antibodies were depleted of IgG specific for PR3, indicating that binding and activation are not related to PR3, which is the main cytoplasmic ANCA found in patients with WG. The choice of EC for this study was based on our recent published observation12 that patients with WG displayed highest frequencies of AECA against kidney EC (71%), as compared with the other relative target EC (e.g., nose 61%, lung 25%), including that the kidney is one of the major target organs during active WG disease.

A first indication of endothelial activation was demonstrated when stimulation by WG IgG triggered a rapid calcium flux response within 5 to 10 s in kidney EC. Therefore, a change in calcium flux is the first feature of cell activation, which initiates and coordinates specific cellular activities after a given stimuli.19,20 Furthermore, the binding of WG IgG induced production of two chemokines: MCP-1 and GCP-2. MCP-1 is an important mediator of monocyte and T cell infiltration of tissues in various inflammatory diseases, including atherosclerosis and rheumatoid arthritis.21 GCP-2, however, promotes mainly neutrophil migration and activation, whereas monocytes, eosinophils, and lymphocytes all are unresponsive to GCP-2.22 These chemokines may play a role in the lesions of WG granulomas that are mainly composed of cells of the monocyte/myelocyte lineage together with lymphocytes, although many other chemokines and cytokine profiles may be implicated in the recruitment of immune cells.23 It is interesting that both IL-8 and GRO-α levels by HKMEC were decreased upon WG IgG stimulation. The biologic significance of this observation is not known. It also is not known whether the responses induced by AECA in HKMEC are specific for this cell line. WG AECA that are known to bind other EC, such as nasal and lung EC, need to be tested to establish the cell/organ specificity of the AECA responses.

Another sign of activation after WG IgG AECA stimulation was observed by the upregulation of two stress- and inflammation-related molecules—MICA and VAP-1—on the surface of kidney EC. It has been demonstrated that MICA is upregulated by heat shock and viral infection but not by IFN-γ.24 As mentioned previously, MICA acts as a ligand for natural killer cells CD8+ T cells and γδ T cells, which express the receptor NKG2D.18 In this study, we demonstrate the infiltration of inflammatory cells such as NKG2D+, CD8+, and γδ T cells in the kidney biopsies of patients with WG during active disease. In CD8αβ T cells, the NKG2D receptor functions as a co-stimulatory molecule25 augmenting the T cell receptor (TCR) stimulation, but in NK cells, the NKG2D receptor leads to

Figure 4. The JNK inhibitor SP600125 and the JNK peptide inhibitor 1 at various concentrations (as indicated) significantly inhibited phosphorylation of c-Jun and ATF-2. Phosphorylation of c-Jun (A and B) and ATF-2 (C and D) was quantified by densitometric analysis using Imager (Quantity 1, version 1.2) and normalized to control levels (AECA-treated cells without the two inhibitors) arbitrarily set to 100%. Data are means ± SD (n = 3). *P < 0.05, **P < 0.001 versus nontreated cells.
cell-mediated cytotoxicity and cytokine production, all in the absence of any additional activation signal.25

Patients with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and WG have increased levels of CD8+CD28null T cells expressing NKG2D, as compared with matched healthy individuals. The expression of NKG2D could present a mechanism through which TCR activation can be increased and through which the threshold for T cell activation by self-antigens can be lowered. Because NKG2D is constitutively expressed, the upregulation of its stress-induced ligands MICA/MICB may be the set-off event leading to the breakdown of tolerance that could further contribute to chronic inflammation or autoimmunity.

Because VAP-1 is an adhesin and also an enzyme producing potent inflammatory mediators such as hydrogen peroxide, aldehyde, and ammonium, its upregulation and translocation to the EC surface may result in a multitude of effects. It may directly increase binding of leukocytes to inflamed vasculature in kidney and their migration into the tissue. Moreover, hydrogen peroxide produced by VAP-1 may upregulate other adhesion molecules and molecules involved in oxidative stress pathways, further facilitating the inflammatory reaction. Aldehyde, however, leads to nonenzymatic glycosylation of proteins and formation of advanced glycation end products, which are harmful for the vasculature.26,27

Several studies have attempted to characterize the nature of the target antigen(s) expressed on HUVEC recognized by AECA in sera from patients with WG.28,29 H-lamp-2 was identified as a potential PMN-associated autoantigen in ANCA-associated vasculitis.30 An interesting aspect of this study was that apparently autoantibodies against H-lamp-2 reacted not only with PMN but also with an unidentified protein on renal microvascular EC. However, in our study, no cross-reactivity of AECA with neutrophils was observed.

In this study, WG IgG precipitated a band with high intensity of 190 to 200 kD and two bands of 70 to 73 and 50 to 53 kD using kidney EC. Further molecular identification and characterization of the immunoprecipitated proteins are under progress at our center.

At the molecular level, we found that JNK was selectively phosphorylated after stimulation of HKMEC with AECA WG IgG. The major function of MAPK signaling pathways is to

Figure 5. (A) A representative picture (n = 3) of flow cytometric analysis demonstrating that WG IgG AECA induce a strong expression of the stress-induced molecule MICA (gray line) on HKMEC. However, various concentrations (as indicated) of the JNK-specific inhibitor SP600125 significantly abrogated the expression of MICA on HKMEC in a dosage-dependent manner. No binding of normal IgG to HKMEC or any effect of inhibition with SP600125 on HKMEC was observed. (B) Immunocytochemical analysis of HKMEC treated as described previously gave similar results.
regulate gene expression in response to extracellular stimuli.31 Because stress signals are mediated mostly by the JNK1/2 and p38 MAPK pathways,10 we examined the phosphorylation status of c-Jun and ATF-2, substrates for both JNK1/2 and p38,10 after AECA stimulation and found that there was a marked increase in the phosphorylation of c-Jun and ATF-2 through the JNK but not p38 pathway. We demonstrate that at least two important effects of increased JNK and ATF-2 phosphorylation after AECA stimulation are to induce production of chemokines MCP-1 and GCP-2 and expression of the stress-related molecule MICA, because JNK-specific inhibitors significantly decreased production of the two chemokines and completely abrogated the surface expression of MICA. However, VAP-1 expression was slightly or not affected by these inhibitors, indicating that downstream signaling of JNK does not affect VAP-1 expression and further suggesting that transcription factors activated by JNK signaling do not regulate VAP-1 gene transcription. SAPK/JNK and VAP-1 may be potential therapeutic targets in vasculitis.

In vasculitis, AECA may exert their pathogenic effects in various ways. AECA may be one of the several factors involved in the initiation of WG and play an important role during the primary stages of the disease. The binding of AECA in WG to EC may directly trigger local production of inflammatory cytokines or alternatively might be instrumental in inducing endothelial phenotype changes, resulting in inflammation or facilitation of cellular infiltration.3,32–37 AECA have been shown to recognize constitutive EC surface proteins and to fluctuate with the clinical activity of the disease.32,33 Evidence exists that AECA from WG sera can modulate EC function. Incubation of HUVEC with AECA IgG from patients with WG upregulated the expression of E-selectin, intercellular adhesion molecule-1, and vascular cellular adhesion molecule-1 (CD106).34,38 Expression of these surface molecules indicates endothelial activation and leads to leukocyte adhesion and diapedesis during an inflammatory response. AECA bound to HUVEC also induced a parallel increase in cytokine (IL-1 and IL-6) and chemokine (IL-8 and MCP-1) production that seems to support the upregulation of the mentioned adhesion molecules in an autocrine manner.32 The pathogenic role of AECA is further highlighted by an experimental model of induced systemic vasculitis (SV) wherein mice immunized with IgG AECA from a patient with WG displayed histopathologic signs of both renal and pulmonary vasculitis.7 Furthermore, patients who were positive for AECA but negative for ANCA were found to be at risk for a clinical relapse.33,36 Together, these data support a pathogenic role for AECA.

Table 4. Demographic and clinical characteristics of patients with active WG

<table>
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<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Years of Disease</th>
<th>Main Tissue Involvement</th>
<th>Treatment</th>
<th>PR3 (U/ml)</th>
<th>Hb (g/L)</th>
<th>CRP (mg/L)</th>
<th>Serum Creatinine (μmol/L)</th>
<th>WBC Count (Cells/ml)</th>
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<td>1c</td>
<td>F</td>
<td>75</td>
<td>2</td>
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<td>39</td>
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<td>2</td>
<td>M</td>
<td>74</td>
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<td>Nose, kidney</td>
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aCRP, C-reactive protein; ENT, ear, nose, throat; Hb, hemoglobin; WBC, white blood cell.
bValues are given at time of blood sampling.
cPatients from whom kidney biopsies were obtained.
Binding of AECA to kidney EC may result in the possible recruitment of inflammatory cells via MICA and VAP-1 and production of chemokines. This may lead to direct glomerular EC injury or altered glomerular barrier function and glomerulonephritis. WG AECA may play a potentially important role in the pathogenesis of WG.

CONCISE METHODS

Cell Cultivation
HKMEC were selected for this study because the kidney is a major organ affected in patients with WG (85%). HKMEC were freshly isolated from one kidney donor and cultivated as described by us previously. In a similar manner, HKMEC from two additional healthy kidney donors were isolated to ascertain reproducibility of the results obtained in this study.

HUVEC were used as control cells (Clonetics, Stockholm, Sweden). Permission for this study was granted from the local ethics committee. Capillary formation in matrigel and morphologic characterization of HKMEC were performed by light microscopy. Scanning electron microscopy was used to detect fenestrae in the EC.

Generation of VAP-1– and MICA-Expressing Cells
VAP-1 transfectants were produced by transfecting Ax cells (a rat PLN HEV-derived EC line) with VAP-1 cDNA in a eukaryotic expression vector pcDNA3, by lipofection and selecting geneticin-resistant colonies. MICA*005 expressing cells were generated as described previously.

Figure 7. (A and B) Immunohistochemical analysis shows positive staining (black) for MICA in peritubular endothelium and glomerular EC and epithelial cells of the kidney and also intense staining (black) for VAP-1 in peritubular and tubular endothelium in patients with WG during active disease. (C) Control staining with only the secondary antibody. For expression of VAP-1 in normal kidney specimens see Koskinen et al. (D through F) WG kidney sections also showed the presence of infiltrating NKG2D+ cells, CD8+ T cells, and γδ+ T cells around tubular and glomerular capillaries (G through I), which were not detected in the normal control biopsy sections. Magnifications: ×40 in A through G; ×20 in H and I.
Preparation of IgG Fraction and Depletion of Anti-PR3 IgG and Immune Complexes
Sera from 13 patients who had WG (at debut, before treatment; Table 4) and were known to have AECA reactive against HKMEC (on the basis of our previous results) were pooled. Total IgG fractions were isolated using goat anti-human IgG (Fc-chain specific) agarose beads (Sigma Aldrich Sweden AB, Stockholm, Sweden) according to standard procedure. The total IgG concentration was approximately 25 mg/ml. Normal control IgG (11 mg/ml) ChromPure Human IgG was purchased from Jackson ImmunoResearch (West Grove, PA).

For anti-PR3 absorption, 650 µg of PR3 antigen (Wieslab AB, Lund, Sweden) was coupled to a 1-ml HiTrap NHS-activated HP affinity column (Amersham Bioscience AB, Uppsala, Sweden) according to the protocol supplied by Amersham Bioscience AB. The efficiency of anti-PR3 depletion was evaluated by measurement of the ANCA activity by anti-PR3 ELISA (PR3-ELISA; Wieslab AB) according to the manufacturer's protocol, and IgG concentration was determined by standard Mancini method. ANCA activity was determined before and after absorption of anti-PR3 IgG. Also, normal IgG was tested for ANCA activity.

For ensuring further that the IgG fractions did not contain immune complexes, the IgG fraction was purified using a fast protein liquid chromatography (Pharmacia, Stockholm, Sweden). Endotoxin concentration in the isolated PR3-depleted WG IgG fraction was 5 pg/mg IgG.

Binding of Purified PR3-Depleted IgG to HKMEC by Flow Cytometry
Flow cytometric analysis as described previously was performed to demonstrate that purified PR3-depleted IgG fractions retained the capacity to bind HKMEC. Similarly,HUVEC primary cells, human renal epithelial primary cells (Clonetics), and PMN (n = 4) were used to examine whether WG IgG were directed against other endothelial, epithelial, and lymphocyte antigens. Stained cells were analyzed by a flow cytometer (FACSsorter; Becton Dickinson, San Jose, CA). Using initial experiments, a concentration of 0.5 mg/ml WG IgG was estimated as optimal for activation of HKMEC and was used for all subsequent analyses. For substantiation of the specificity of PR3-depleted WG IgG, dosage responses were studied in some of the following analyses.

Measurement of Ca²⁺ Flux in HKMEC
The ability of PR3-depleted IgG to mobilize calcium flux, which is a primary feature of cell activity, was tested as described previously using HKMEC and HUVEC. The cells were incubated with 5 µM Fura 2-AM (Calbiochem, La Jolla, CA) and 0.3 mg/ml pluronic F-127 (Sigma-Aldrich Sweden AB) in HBSS (Ca and Mg included; Invitrogen, Stockholm, Sweden) with 10 mM HEPES for 30 min at 37°C and 5% CO₂. The results were recorded as the ratio of fluorescence between 340 and 380 nm, calibrated, and calculated with commercially available software (Miracl; Life Science Resources Ltd., Cambridge, UK) according to the manufacturer's recommendations. After approximately 10 s of recording, 50 µl of PR3-depleted WG IgG or normal IgG was added to the cells in final concentrations of 1.0, 0.5, 0.3, and 0.1 mg/ml. The total recording time was 200 s.

Measurement of Chemokine Levels in Unstimulated and PR3-Depleted WG IgG-Stimulated HKMEC
Both neutrophils and monocytes have been implicated in the pathogenesis of WG. Therefore, we tested the presence of chemokines and cytokines that may activate and recruit these cell types. HKMEC were stimulated either with 0.5, 0.3, 0.2, or 0.1 mg/ml normal IgG or WG IgG for 16 h. One set of cells was left unstimulated. Supernatant levels of MCP-1, IL 8, GCP-2, GRO-α, TNF-α, and IFN-γ were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Immunocytochemistry for VAP-1 and MICA
For detection of MICA expression, HKMEC were grown separately in 96-well chamber culture dishes and stimulated with either 0.5 mg/ml normal IgG or PR3-depleted IgG for 16 h. One set of cells was left unstimulated. We previously found that VAP-1 expression is lost on culture of EC; therefore, all experiments for detection of VAP-1 were performed using only freshly isolated HKMEC. After IgG stimulation, the cells were stained with mAb against VAP-1 (clone TK8–14) and MICA673 (dilution 1:50; Immunex Corp., Seattle, WA), and the procedure was as before. Transfected VAP-1 and MICA*005 cells served as positive controls.

Western Blotting for Detection of Proteins Recognized by AECA
For identification of the autoantigen(s) that are recognized by PR3-depleted IgG, HKMEC were labeled and immunoprecipitated according to standard procedure. The biotin-labeled proteins were purified by immunoprecipitation (with 10 mg/ml normal or WG IgG) and then electrophoretically separated on an 8% resolving gel. The chemiluminescence signal was visualized by exposure on x-ray film (Hyperfilm ECL; Amersham Bioscience AB).

Western Blotting for Studying Signaling Pathways
For investigation of the signal transduction pathways in HKMEC after PR3-depleted AECA IgG stimulation, the intracellular signaling proteins NF-kB p65, total SAPK/JNK, phospho-SAPK/JNK, total p38, phospho-p38 MAPK, total p44/42, phospho-p44/42 MAPK, pELK-1, phospho-c-Jun, and phospho-AFT-2 were studied. All antibodies were purchased from Cell Signaling Technology (Stockholm, Sweden). The JNK-specific inhibitor SP600125 and the JNK peptide inhibitor 1 were purchased from Calbiochem (San Diego, CA).

HKMEC were stimulated with either 0.5 mg/ml normal IgG or PR3-depleted IgG for 25 min. One set of cells was left unstimulated. TNF-α–stimulated cells were used as positive control. For the JNK inhibitor studies, HKMEC were preincubated with the specific inhibitors at various final concentrations of 20, 30, and 50 µM for 30 min followed by addition of 0.5 mg/ml WG IgG AECA for 25 min. Culture supernatants were tested for chemokine production of MCP-1 and GCP-2 as described previously. Cell lysates, prepared as described previously, were immunoblotted with the previously mentioned antibodies (all 1:200) using the standard SDS-PAGE and Western blots analysis.
Immunohistochemical Staining of Kidney Sections
Frozen kidney sections (5 μm) from patients who had WG (n = 6; see Table 4 for details) with active and confirmed kidney involvement were stained with anti–VAP-1, anti-MICA, anti-CD8, anti-γδ TCR (Becton Dickinson, Stockholm, Sweden), and anti-NKG2D (Serotec, Oxford, UK) according to standard immunohistochemical procedure as described previously. Normal kidney biopsy specimens were used for comparison.

Statistical Analyses
All values are expressed as means ± SD unless otherwise indicated. P < 0.05 was considered to be statistically significant. Differences among four groups were analyzed by ANOVA using Kruskal-Wallis test. The statistical analysis was performed using statistical software SAS 9.1.3 (SAS Institute, Cary, NC).

ACKNOWLEDGMENTS
This study was financed by grants from the Medical Research Council (K2005–06X-14004-02B), The Konung Gustaf V’s 80 Year Foundation, the Reumatikerförbundet Foundation, and the Sunds Foundation to S.S.-H.

We thank Dr. Kalle Söderström for the MICA cell line.

DISCLOSURES
None.

REFERENCES
ANCA positive vasculitis are associated with disease activity. Clin Exp Immunol 139: 569–574, 2005