@ Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis

Alexander Woywodt, Frank Streiber, Kirsten de Groot, Heide Regelsberger, Hermann Haller, Marion Haubitz

Summary

Background Histological findings in small-vessel vasculitis associated with antineutrophil cytoplasmic antibodies (ANCA) suggest that damaged endothelial cells undergo necrosis and detachment from the basement membrane. We postulated that isolation of these cells from peripheral blood might provide a novel marker of the disease and elucidate pathogenetic events.

Methods 18 patients with active ANCA-associated vasculitis, 20 patients in remission, 20 healthy controls, 12 patients with infection, and 12 patients with glomerular disease not associated with ANCA were studied. Endothelial cells were isolated from peripheral blood by use of Dynabeads coated with antibodies against CD146, and were stained for von Willebrand factor (vWF), CD31, and Ulex Europaeus lectin 1 (UEA-1). Tissue-factor immunocytochemistry and assays for markers of apoptosis and necrosis were also done.

Findings Few circulating endothelial cells were seen in healthy controls (0–20 cells/mL, median 5 cells/mL), patients with infection (0–16 cells/mL, median 8 cells/mL), and patients with non-ANCA glomerulonephritis (0–21 cells/mL, median 4 cells/mL). By contrast, large numbers of circulating endothelial cells were detected in patients with active vasculitis (20–5700 cells/mL, median 136 cells/mL, p<0·0001 when compared with healthy controls). Cell numbers fell substantially during 6 months of successful immunosuppressive treatment among those with active disease. Patients in remission had moderately raised cell numbers (0–60 cells/mL, median 16 cells/mL). 84% of cells obtained from patients with active disease stained positive for annexin/propidium iodide and 86% stained tissue factor positive, indicating a necrotic and procoagulant phenotype.

Interpretation Circulating endothelial cells are a novel marker of active ANCA-associated small-vessel vasculitis. The clinical use of this tool and the pathogenic mechanisms leading to these findings require further investigation.

Introduction The discovery of antineutrophil cytoplasmic antibodies (ANCA) not only heralded the advent of a novel laboratory marker for a subset of small-vessel vasculitides, but also provided a new pathogenetic concept. The contact of primed neutrophils with endothelial cells, possibly mediated by ANCA, is regarded as a key event. In this regard, the interaction between neutrophils and ANCA has been the subject of much study whereas endothelial cells have received less attention. Histological findings in ANCA-associated small-vessel vasculitis suggest that endothelial cells undergo necrosis and detachment from the basement membrane and are released into peripheral blood. As yet, however, no attempts have been made to isolate these cells from blood, although methods to do so have recently become available. We became interested in these cells for three reasons.

First, a close correlation might exist between disease activity and the extent to which endothelial cells and debris are released from lesions of ongoing or recent inflammation. Therefore, circulating endothelial cells could be a disease activity marker. Second, endothelial damage should be less prominent in many disorders that mimic systemic vasculitis, such as infection or connective tissue disease, suggesting that an assay of circulating endothelial cells might help with the differential diagnosis. Finally, research into the pathogenesis of vasculitis is hampered considerably by problems in obtaining endothelial cells from patients. In particular, these cells are few and difficult to isolate from biopsy specimens. Therefore, most research in this area has been done in human umbilical-vein endothelial cells that can differ substantially from their mature counterparts.

We sought to isolate endothelial cells from peripheral blood of patients with active and quiescent ANCA-associated vasculitis, to characterise the cell phenotype, and to relate cell numbers to disease activity.

Methods Patients and controls

We investigated 38 patients with biopsy-proven ANCA-associated small-vessel vasculitis. 22 patients had Wegener’s granulomatosis and 16 had microscopic polyangiitis. Disease activity was assessed according to the Birmingham vasculitis activity score (BVAS). 18 patients (15 with Wegener’s granulomatosis, three with microscopic polyangiitis; 11 male, age 30–79 years, median 62·5 years) had clear-cut evidence of active disease (BVAS scores 7–39, median 16). 15 of these 18 patients were eligible for follow-up during 6 months of immunosuppressive treatment; the other three were followed up for 1–5 months. 20 patients (seven with Wegener’s granulomatosis, 13 with microscopic polyangiitis; 14 male, age 26–72 years, median 54·5 years) were in remission (BVAS scores 0–1, median 0).
We also studied three patients with isolated granulomatous ear, nose, and throat (ENT) disease due to Wegener’s granulomatosis, and 20 healthy controls (age 22–71 years, median age 37·5 years). Six patients with pneumonia and six with intra-abdominal sepsis (age 37–81 years, median age 51·5 years; median C-reactive protein concentration 80 mg/L) were examined as infectious-disease controls, and 12 patients (nine male, three female; age 29–73 years, median 43·5 years) with non-ANCA associated glomerulonephritis served as renal-disease controls.

Procedures
To isolate circulating endothelial cells, we used M-450 Dynabeads (Dynal, Hamburg, Germany) coated with murine anti-CD146 antibody (Biocytex, Marseilles, France). CD146 is expressed almost exclusively on mature endothelial cells, the exception being some tumour cell lines. 15 mL peripheral blood was obtained with a 21G butterfly needle and the first 7·5 mL discarded. We had previously shown that these measures were sufficient to avoid any influence of venepuncture, that there was no influence of arterial versus venous sampling, and that diurnal variations could be excluded (data not shown). 2 mL blood was diluted with 2 mL buffer and 20 µL FcR blocking agent (Miltenyi, Gladbach, Germany) and incubated with 100 µL anti-CD146-coated beads (1·4×10⁸ coated beads/mL) for 30 min at 4°C. Cells bound to anti-CD146-coated beads were separated from blood in a magnet, washed, dissolved in 100 µL buffer, mixed with acridine, and counted in a Nageotte chamber (Brand, Wertheim, Germany). To serve as positive controls, we mixed circulating endothelial cells with fresh human umbilical-vein endothelial cells, and tested for non-specific binding to Dynabeads, isothiocyanate-coupled antibodies against rabbit IgG were used as antibody to prevent this problem. CD3, CD8, CD14, and CD45 (Dianova, Hamburg) staining of our isolated cells was negative (data not shown). We also did side-by-side assays with M-450 Dynabeads coated with human antibodies against mouse IgG but without anti-endothelial antibody. These assays were entirely negative, suggesting that non-specific binding to Dynabeads did not occur. To further challenge the specificity of the technique, we mixed blood with peripheral-blood mononuclear cells and granulocytes at various concentrations and tried to isolate circulating endothelial cells; these controls were also negative. To ensure that all isolated cells were indeed endothelial cells, we stained them with antibodies to von Willebrand factor (vWF), CD31, and Ulex Europaeus lectin 1 (UEA-1), as well as with acridine. Rabbit antibodies against human vWF, and fluorescein-isothiocyanate-coupled antibodies against rabbit IgG were from Boehringer, Mannheim, Germany; sheep antibodies against human CD31, and fluorescein-isothiocyanate-coupled antibodies against sheep IgG were from Binding Site, Heidelberg, Germany. UEA-1 was from Linaris, Wertheim, Germany. Eventually, we were able to identify cells on the basis of morphology and adherence to beads alone, so that concurrent immunocytochemistry was unnecessary; endothelial cells were larger than other blood cells, had a well-delineated round or oval cell shape, and more than five beads were attached.

Results
We examined cell morphology and did immunocytochemistry with cytospin preparations, using an alkaline phosphatase/anti-alkaline phosphatase immunocytochemistry, ×1000. We did TUNEL staining in accordance with standard techniques and reagents (Roche, Basle, Switzerland). We used the apoptosis detection kit (Roche, Basle, Switzerland) in accordance with instructions. To exclude any effect of the isolation procedure on cell viability, we examined human umbilical-vein endothelial cells diluted in blood obtained from healthy controls. The apoptosis rate of these cells was less than 5% before and after immunomagnetic isolation; necrotic cells were undetectable. For culture assays, we suspended cells isolated from peripheral blood of two patients with active vasculitis in culture medium, then seeded onto two-well culture slides, and incubated for 24, 48, and 72 h. After fixation, vWF immunocytochemistry was done as for cyto spin preparations.

Statistical analysis
Unpaired Mann-Whitney testing (two-sided) was used for comparison of cell numbers between patients and controls after the Kruskal-Wallis test had been applied to show significant differences between patients and controls. Friedman’s test was used to show that cell numbers at 0, 1, 3, and 6 months were different, and paired Wilcoxon’s testing (two-sided) was used for comparison of these cell numbers.

Role of the funding source
The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
Using immunomagnetic isolation, we identified a well-defined population of cells in peripheral blood of patients with ANCA-associated vasculitides; these cells reproducibly stained positive for vWF, CD31, and UEA-1 which was regarded as sufficient proof of their
endothelial origin. These circulating endothelial cells were larger than other blood cells (10–50 μm in length) and had an oval or round shape, as shown in figure 1. In patients with active vasculitis, larger aggregates were also seen, as were small cellular particles and membrane fragments (figure 1). These particles carried CD146-coated beads and stained positive with all the endothelial cell markers. We therefore regarded them as endothelial cell fragments. Such fragments were never seen in any of the controls.

86% of the circulating endothelial cells stained positive for tissue factor, with homogenous staining of the cytoplasm, as shown in figure 1. All cells studied with the TUNEL assay were negative, as shown in figure 2. However, 84% of endothelial cells stained positive for annexin and propidium iodide, consistent with a necrotic rather than an apoptotic phenotype (figure 2). Only 16% stained with annexin alone. Correspondingly, we were unable to culture endothelial cells from two patients with active vasculitis (data not shown).

Very few endothelial cells (0–20 cells/mL, median 5 cells/mL) were detected in healthy controls. Similar cell numbers were seen in patients with infection (0–16 cells/mL, median 8 cells/mL, not significantly different from healthy controls p=0.185) and those with non-ANCA glomerular disease (0–21 cells/mL, median 4 cells/mL, no significant difference compared with healthy controls p=0.118). By contrast, large numbers of circulating endothelial cells were seen in blood obtained from patients with active ANCA-associated vasculitis (20–5700 cells/mL, median 136 cells/mL, mean 871 cells/mL [SD 1741], p<0.0001 when compared with healthy controls). There was a highly significant, albeit moderately strong, correlation between cell numbers and BVAS scores when all patients with quiescent and active vasculitis were included, the latter both at baseline and during follow-up (Spearman's rank correlation, r=0.704, p<0.0001, n=86).

During treatment, one patient had a minor ENT relapse without any change in cell numbers; one patient had gram-negative sepsis due to cholecystitis and cholangitis and died despite surgery; notably, cell counts remained low despite continuing sepsis. Patients in clinical remission had moderately raised endothelial-cell numbers (median 16 cells/mL [range 0–60 cells/mL], mean 23.3 cells/mL [SD 20]), when compared with healthy controls (p=0.0192), but significantly fewer than patients with active vasculitis (p<0.0001). Three patients with granulomatous disease due to Wegener’s granulomatosis but without evidence of vasculitis had 24, 16, and 8 cells/mL.

Figure 2: Apoptosis/necrosis assays in patients with active Wegener’s granulomatosis
A: TUNEL stain with haemalaun counterstaining. B: annexin staining of a circulating endothelial cell. C: propidium staining of same endothelial cell as in B.

Figure 3: Cell numbers in patients with active and quiescent ANCA-associated vasculitis, healthy controls, patients with glomerular disease not associated with ANCA, and patients with infection
Data are ranges, IQRs (shaded areas), and medians (horizontal lines within shaded areas).
vasculitis, but their pathogenetic importance remains undefined. Endothelial damage occurring in experimental ANCA-associated small-vessel vasculitis is thought to result at least in part from release of toxic proteins into the circulation. Proteinase-3 is an example of such a target antigen. Its release could result from necrosis or defenestration of the capillary endothelium. However, the precise nature of the mechanism by which this occurs remains to be explained. Endothelial cells have been shown to possess the tissue-factor gene. 

Necrosis is a process by which a cell loses its integrity. It is an inevitable consequence of the death of most cells. Necrosis may be caused by a number of different mechanisms, including damage to the cell membrane, oxidative stress, and apoptosis. Necrosis is characterized by the loss of cell membrane integrity, leading to the release of cellular contents into the extracellular space. This can result in the formation of a necrotic zone around the dead cell, which can attract immune cells to the site of injury.

Figure 4: Cell numbers during the course of immunosuppressive therapy in patients with active disease

![Graph showing cell numbers during the course of immunosuppressive therapy](image)

We found that patients with active ANCA-associated vasculitis have high numbers of circulating endothelial cells, compared with patients with quiescent disease or limited ENT disease, patients with other granular disorders, patients with infection, and healthy controls. Furthermore, the endothelial cells have apparently undergone necrosis and thereby have probably been released because of a direct, active, pathogenic mechanism.

Severe endothelial damage with invasion of neutrophils and fibrinoid necrosis is a salient feature of ANCA-associated small-vessel vasculitis. Interaction between activated endothelial cells and primed neutrophils seems to be crucial to the pathogenesis of these lesions. Yet the precise mechanisms of endothelial damage and the role of ANCA remain poorly understood. The assumption that ANCA are pathogenic is still debated, and binding of ANCA to endothelial cells remains unproven. In particular, expression of protease-3, a target antigen for ANCA, by endothelial cells has been refuted. Endothelial damage may be caused by ANCA-induced release of toxic enzymes from neutrophils. Antienzyme antibodies have also been detected in ANCA-associated small-vessel vasculitis, but their pathogenetic importance remains to be explained. The mechanisms of endothelial damage, ultrastructural features of this process are well described and culminate in denudation of the basement membrane and thrombosis.

The fate of these endothelial cells has not previously been elucidated and nor have circulating endothelial cells been studied in vasculitis. Raised concentrations of endothelial proteins such as von Willebrand factor (vWF) and thrombomodulin in serum have been documented previously, although whether release of these proteins is due to endothelial activation or frank necrosis is unclear. Circulating endothelial cells have already been detected in other vascular disorders, including those related to thrombosis, infection, and immunity. Earlier attempts to use detection of circulating endothelial cells to diagnose such disorders date from the 1970s, and focus on morphological features of endothelial cells in peripheral blood smears. Later, immunocytochemistry with antienzyme antibodies was used to detect circulating endothelial cells. Recent work has introduced immunomagnetic isolation as a new technique to isolate endothelial cells from peripheral blood.

Our data suggest two lines of interpretation. First, the number of circulating endothelial cells seems to be a promising new laboratory marker of small-vessel vasculitis; other markers, particularly ANCA testing, have performed with poor specificity and sensitivity. With a cutoff of 25 cells/mL for a positive test result, the test for endothelial cells had good specificity and sensitivity. In patients with active disease, cell numbers declined during treatment, and patients in remission had much less raised cell counts. Three patients with limited granulomatous disease also had fewer circulating endothelial cells than patients with active vasculitis. We studied disease controls with various renal disorders and infection and found that our test was able to discern patients with active vasculitis from these patients. These results give us confidence in our interpretations.

Second, we were interested to identify necrosis, rather than apoptosis, in circulating endothelial cells, and were able to show that the isolation procedure itself did not induce necrosis in endothelial cells. The role of these necrotic cells is unclear. Necrotic, but not apoptotic cells, have been shown to induce inflammatory mechanisms via the nuclear factor (NF)-κB and the Toll-like receptor 2 pathway, and to increase antigen presentation by macrophages. Moreover, we showed tissue-factor expression, indicating that these cells acquire a procoagulant phenotype during the process of necrosis and detachment. Nevertheless, in an in-vitro system, apoptotic human umbilical-vein endothelial cells were also found to have procoagulant properties. The tissue-factor gene possesses NF-κB and Toll-like receptor 2 binding sites. Tissue-factor expression might be pivotal to vasculitis, since tissue factor links proinflammatory mechanisms with thrombosis. Endothelial tissue-factor expression might thus be a crucial event in vasculitis. We found earlier that release of proteinase 3 and elastase from neutrophils induces endothelial-cell tissue-factor expression. Fibrin deposition in turn induces tissue-factor expression in endothelial cells, thereby leading to a vicious circle of inflammation and thrombosis.

We are curious about how necrotic endothelial cells might undergo pulmonary capillary passage. The cell number originally released from the lesions is likely to be far exceeded by the number detected here and therefore a substantial proportion might be cleared in pulmonary capillaries. One approach to clarify this issue might be to sample blood from affected and non-affected tissues concurrently, and compare cell numbers with those seen in peripheral blood.

Our study has limitations. First, our findings need to be confirmed in larger numbers of patients with longer follow-up. Therefore, we recommend that our results be considered preliminary.
follow-up. Patients who relapse and those who have sustained infectious complications during treatment need to be studied as well. Other vasculitides should also be examined, although we do not regard our findings as specific to ANCA-associated disease. Widespread use of this marker is also impeded by the fact that our method requires substantial experience. Standardisation may be a problem, since techniques differ with respect to their limits of detection and normal values. In our study, cell numbers in healthy controls were somewhat higher than reported previously. A simpler assay would be of great use, and we are currently exploring several approaches.

Contributors
A Woywodt developed the protocol for isolation of circulating endothelial cells and wrote the manuscript. F Streiber did the assays and collected clinical data. K de Groot was responsible for the clinical care of some of our patients. H Regelsbrger isolated circulating endothelial cells and did immunocytochemistry. H Haller supervised the study. M Haubitz had the initial idea, supervised the study, and contributed to the final version of the manuscript.

Conflict of interest statement
A Woywodt delivered a talk for Dynal, the manufacturer of Dynabeads, at the Dynal Workshop held in Hannover, Germany, in April 2001, and received financial compensation on this occasion.

Acknowledgments
We thank Friedrich C Luft, Division of Nephrology, Franz-Volhard-Clinic, Berlin, for help with the manuscript, and Ursula Goebel for referral of patients. We also thank Astrid Borgnes, Dynal, Norway, for invaluable help with the technique. Finally, we thank Alena Herrmann, Ferdinand Bahlmann, and Maik Schroeder, for helpful discussion.

This work was funded by an intramural research grant bestowed by the Dean for Postgraduate Research, Hannover Medical School. It was presented in part to the American Society of Nephrology at the World Congress of Nephrology, San Francisco, CA, USA, October, 2001.

References