Immunologic Detection of Endothelial Cells in Human Whole Blood

By Rosella Sbarbati, Martin de Boer, Mario Marzilli, Maria Scarlattini, Giuseppe Rossi, and Jan A. van Mourik

In this report we show that human endothelial cells (EC) can be detected in circulating blood by means of the EC-specific monoclonal antibody (MoAb) designated as CLB-HEC 19 and expressed quantitatively as number of cells per milliliter of whole blood. We first developed a method that was able to recover cultured human EC added to whole blood by Percoll density gradient centrifugation. The final recovery of the EC was 91.6% (SE = 0.65%). The EC were identified in the gradient by immunofluorescence with the MoAb CLB-HEC 19. This method was then applied to the separation and characterization of EC or EC remnants from the whole arterial and venous blood taken from two groups of patients subjected to heart catheterization. Firstly, a preliminary blood screening of random samples was performed in a group of eight patients (group I) using a scoring evaluation for the presence of EC and the results were expressed as positivity index. Secondly, the complete blood screening of a group of ten patients (group II) was performed for the detection of immunofluorescent cells and the results were expressed as number of EC per milliliter of whole blood. Our results show in both group I and II a significant presence of EC in the blood after catheterization compared with their basal values. The minimal detectable concentration of EC was 0.06 cells/mL (SE = 0.057) of whole blood. We consider this technique as a suitable clinical test for the detection of EC injury in cardiovascular pathology.

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MATERIALS AND METHODS

Isolation and culture of human umbilical vein EC (HUVEC). HUVEC were isolated from umbilical cords collected in phosphate-buffered saline (PBS). The cords were processed from day 0 to 3 after delivery following a standard protocol. HUVEC were cultured in 75-cm² culture flasks precoated with fibronectin in a complete culture medium (CM) composed of culture media 199 and RPMI 1640 (GIBCO, Paisley, U.K.), 20 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 100 U/mL penicillin-streptomycin, 5 µg/mL fungizone, and 20% pooled human serum from 20 healthy donors. Cells grown at confluence (second or third passage) were trypsinized (0.05% trypsin/0.02% EDTA [wt/vol]) or harvested with a cell scraper and resuspended in complete medium before Percoll density gradient centrifugation (see below).

Preparation of Percoll suspensions and HUVEC isopycnic centrifugation. Two Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) suspensions with a specific gravity of 1.050 and 1.10 g/mL, respectively, were prepared as previously described, with some modifications. The specific gravity of 1.050 g/mL was obtained by mixing 34.5 mL of stock Percoll suspension, 45.5 mL of Earle’s medium, 10 mL of pooled human serum, and 10 mL of 3.8% (wt/vol) sodium citrate. The specific gravity of 1.10 g/mL was obtained by mixing 76.6 mL of stock Percoll suspension, 3.4 mL of Earle’s medium, 10 mL of pooled human serum, and 10 mL of 3.8% (wt/vol) sodium citrate. The two Percoll suspensions were mixed with a proportioning pump to prepare linear gradients with specific gravity ranging from 1.050 to 1.10 g/mL.

A 2-mL HUVEC suspension was prepared. Cells were counted by a Coulter counter (Coulter Electronics, Dunstable, UK) and divided into two portions of 1 mL each. The first portion was mixed with 4.5 mL of blood collected in 0.5 mL of 3.8% (wt/vol) sodium citrate and layered on top of a linear Percoll gradient. As a control, the other portion was mixed with 5 mL of CM and layered on top of a second linear gradient. Tubes were centrifuged for 10 minutes at 1000g at room temperature and fractions of 2 mL were collected. Cells per fraction were counted in the presence of 0.02% (wt/vol) saponin + 0.0001% (wt/vol) Triton X-100 (Pierce Eurochemie, Oudbeijerland, The Netherlands). The specific gravity in each fraction was measured with a densitometer (DMA 46, A. Paar, Austria).

The cells of the various fractions were seeded on glass coverslips coated with 1% gelatin fixed with 0.5% glutaraldehyde. Indirect immunofluorescence (see below) was performed within 2 days. The different leukocyte subpopulations were previously identified. Once the position of HUVEC specific gravity was determined along the linear gradient, routine separation of HUVEC fractions was performed in a one-step procedure by centrifugation either of...
the HUVEC in blood or of the HUVEC in CM over a Percoll layer of 1.060 g/mL (42.9 mL of stock Percoll suspension, 10 mL of pooled human serum, 37.1 mL of Earle’s medium, 10 mL of 3.8% (wt/vol) sodium citrate).

**Blood sampling.** VB and AB samples were obtained at the beginning and at the end of routine cardiac catheterization procedures. Catheterization was performed in 18 patients with ischemic heart disease according to the Jundkins method, following premedication with a 10-mg intramuscular Diazepam (Roche, Milan, Italy).

The right femoral artery was punctured using a Seldinger needle and a 0.038” guide wire was introduced into the artery. An 8F pig-tail catheter was advanced over the wire up to the iliac artery, and the wire pulled back. Before advancing the pig-tail catheter into the abdominal aorta, a needle was introduced into the right antecubital vein and blood samples of 4.5 mL were drawn simultaneously from the vein (basal VB [VBV]) and from the artery (basal AB [BAB]) and carefully mixed with 0.5 mL of 3.8% (wt/vol) sodium citrate.

The catheterization procedure was performed, which included left ventricular angiography and selective right and left coronary angiography in multiple projections. The procedure was free from complications in all patients and lasted approximately 30 minutes. At the end of this procedure, venous (final VB [FVB]) and arterial (final AB [FAB]) blood sampling was repeated, being careful to obtain blood simultaneously, from the same vascular district sampled initially.

Controls were performed by drawing venous blood (normal VB [NVB]) from the antecubital vein of seven young healthy people (mean age = 29.6 years, SE = 2.19).

**Processing of clinical samples.** Basal and final whole blood samples were dropped over Percoll layers of 1.060 g/mL, centrifuged at 1000g at room temperature for 10 minutes. The upper cell layer was collected and centrifuged at 400g for 10 minutes. The supernatant was discarded and the cellular pellet, resuspended in PBS and 2% bovine serum albumin (BSA), was sedimented on glass coverslips by means of a cytospin and processed for immunofluorescence (see below).

As a control, HUVEC mixed with whole blood of healthy donors were recovered and processed following the same procedure used for clinical samples.

**Immunofluorescence.** Each staining was performed for 1 hour at room temperature. Slides were washed, fixed with methanol for 10 minutes, washed again, incubated with MoAbs (see below) (ascites, diluted 1:1000 in PBS and 2% BSA), washed, stained with fluorescein isothiocyanate (FITC)-conjugated polyvalent goat antimouse Ig (1:80) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands), washed, and embedded in 87% glycerol in PBS (9:1) and 1 mg/mL para-phenylene diamide (pH = 8.6).

The slides first stained with CLB-HEC19 (see below) were double-stained with a rabbit antihuman serum against the von Willebrand factor (vWF) (1:500) (CLB), washed, and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated polyvalent horse antirabbit Ig (1:80) (CLB).

**MoAbs.** We have previously described the characterization of a series of MoAbs raised against cultured HUVEC.28 One of these antibodies used in our study (CLB-HEC19) recognized an EC-specific plasma membrane protein with an apparent molecular weight of 100,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig 1). This antibody did not react with blood cells, including erythrocytes, lymphocytes, monocytes, granulocytes, and blood platelets, and cultured fibroblasts and smooth muscle cells.
platelet GP Ila and MoAb CLB-RAg 35 directed against the vWF were also used as control antibodies.

Statistical analysis of the clinical samples. Group I blood samples (eight patients): four slides per blood sample were stained with MoAb CLB-HEC 19. They were screened using the fluorescence microscope (Diaphot-TMD-EF, Nikon, Nippon Kogaku KK, Tokyo, Japan) for the presence of EC (patients' blood EC [PBEC]). Four random fields per slide were chosen and each field was assigned figures 0, 1, 2; namely 0, if the field had no fragments; 1, if it contained one fragment; and 2, if it contained more than one fragment. The means of the scored values per blood type were analyzed by the Wilcoxon signed ranks test for paired samples to test the level of significance of the differences between blood types before and after heart catheterization.

Group II blood samples (10 patients): the entire pellet of the Percoll fractions was recovered and sedimented on three slides per blood sample, which were stained with MoAb CLB-HEC 19. Each slide was chosen (blindly and at random) and then screened for the presence of immunofluorescent PBEC. The number of EC per milliliter of whole blood was calculated. The two-tailed paired-sample t-test was used to assess differences between BAB and BVB. Because this difference was statistically not significant, the analysis of variance (ANOVA) for repeated measures was used to test the differences between venous and arterial, basal, and final blood. The two-tailed unpaired-sample t-test was used to test the difference between BVB and NVB. The mean number of cells per milliliter of blood was calculated. The two-tailed paired-sample t-test was used to test the differences between BAB and BVB. Because this difference was statistically not significant, the analysis of variance (ANOVA) for repeated measures was used to test the differences between venous and arterial, basal, and final blood.

RESULTS

The analysis of the cellular content of the fractions in the control experiment where HUVEC were mixed with CM (Fig 2, HUVEC in CM fractions 17 to 24) indicated that the specific gravity of EC ranged from about 1.060 to 1.045 g/mL. The recovery of the EC was about 85%. Fractions 20 to 24 were separately brought into culture and the cells were identified as HUVEC by indirect immunofluorescence (Fig 3b and c). Immunofluorescence pictures of HUVEC in normal culture are shown for comparison (Fig 3a, d, and g).

The cells present in the fractions after separation of the blood sample mixed with HUVEC (Fig 2, HUVEC in blood) showed the presence of three main subpopulations with specific gravities ranging from about 1.085 to 1.075, 1.075 to 1.060, and 1.060 to 1.045 g/mL, respectively. Fractions no. 10, 14, 16, and 19 to 24 (Fig 2) were brought into culture and their cellular content was identified by immunofluorescence. The results show that fractions no. 10, 14, and 16 do not contain EC, while fractions 19 to 24 stain positively with the EC-specific MoAb CLB-HEC19 and with anti-vWF serum (Fig 3c and f). We concluded that the EC are part of the subpopulation of cells with specific gravities ranging from 1.060 to 1.045 g/mL. Fractions containing EC also stained positively for the platelets (Fig 3h). The one-step procedure with a discontinuous gradient of 1.060 g/mL confirmed the results of the linear gradients. The recovery of EC from complete medium was about 92% (Table 1).

The screening of group I patients' blood before and after heart catheterization provided the results shown in Fig 4, where the means of the scores per blood sample are arranged in four ranks. There is a significant difference between the mean positivity indices of the basal and final blood samples (Fig 4). Furthermore, the increase of the positivity index after catheterization does not result to differ significantly between venous and arterial blood. No significant difference was found between the basal values, either. Figure 5 gives the quantitative results obtained from the screening of group II patients' blood. There is no significant difference between BVB and BAB and between NVB and BVB. The mean number of cells per milliliter of both FVB and FAB is significantly higher than that of their respective basal values. Similar to what is found in patients' group I, no significant difference was observed between FVB and FAB, ie, the final effect of catheterization in terms of increase of cell number per milliliter of blood is similar in both VB and AB. The minimal detected EC blood concentration was in NVB, ie, 0.06 cells/mL (SE = 0.057) (Fig 5). No significant difference appears from the statistical comparison of the areas of the two random samples of HUVEC and of PBEC (Table 2). Figure 6 shows some immunofluorescent stainings of EC found in our clinical samples.

DISCUSSION

The method of EC separation from blood presented in this report is based on the difference of the specific gravity of the blood cell subpopulations. Cell identification relies on the use of an EC-specific tool, ie, MoAb CLB-HEC 19 (Fig 1). EC are for the first time detected, identified, and quantified in whole blood from clinical samples. Previous reports' could not discriminate between EC and platelets, owing to the lack of an endothelium-specific antibody and/or to the use of an inadequate cell separation method. Hladovec et al.'s method was repeated by us but we were unable to reproduce the EC recovery from whole blood. The recovery of EC from complete medium was about 92% (Table 1).
Fig 3. Identification of recovered HUVEC by indirect immunofluorescence. HUVEC in normal culture with (a) the EC-specific MoAb CLB-HEC 19 and (d) the polyclonal anti-vWF serum. HUVEC after separation in CM with (b) CLB-HEC 19 and (e) anti-vWF serum. HUVEC after separation from blood cells with (c) CLB-HEC 19 and (f) anti-vWF serum. (g) HUVEC in normal culture stained with an anti-pollen MoAb (negative control). (h) HUVEC after separation from blood cells stained with MoAb 6C9.11 specific for platelet GP IIb/IIIa. The antibody stains a remnant of aggregated platelets between (negative) EC after 2 days in culture. Original magnification ×500.

Fig 6. EC identification by immunofluorescence in clinical samples. The EC-containing fractions are sedimented on glass coverslips by means of a cytocentrifuge and processed for immunofluorescence. PBEC from (b) group I and (d) group II patients’ blood are stained with MoAb CLB-HEC 19. The cells shown in (b) are double stained (e) with a polyclonal antibody against vWF. (c) a FAB sediment shows cell membranes stained with MoAb CLB-HEC 755 and vWF stained with MoAb CLB-RAg 355 in both EC and platelets. (f) is a NVB (negative) sediment stained with CLB-HEC 19. (a) As a control HUVEC detached from the culture flask by a cell scraper and sedimented on glass coverslips are stained with MoAb CLB-HEC 19. Fluorescence microscope: (a and d) MRC 500 laser confocal microscope; (b, c, e, and f) Diaphot-TMD-EF. Original magnification: (a, b, d, and e) ×600; (c) ×400.
Immunofluorescence with endothelial- and platelet-specific MoAbs (see under Materials and Methods) suggested that the cellular yield was mainly constituted by aggregated platelets (unpublished observations). Isopycnic centrifugation in Percoll gradients allows the cells to localize in a position in which their specific gravity equals that of the gradient material. The determination of the specific gravity of the medium in each platelet subpopulation according to their specific gravity: a fraction heavier than 1.075 g/mL containing granulocytes (neutrophils and eosinophils); a fraction with specific density ranging from about 1.075 to 1.060 g/mL containing mostly lymphocytes and basophils; and a fraction lighter than 1.060 g/mL that groups monocytes and platelets. EC, when present in whole blood, belong to the last subpopulation, ie, their specific gravity partially overlaps that of the monocytes and platelets (Fig 2). Therefore, it seems impossible to completely separate these cells by isopycnic centrifugation. Nevertheless, the presence of some monocytes and platelets in the EC subfraction is not a drawback because the use of an endothelium-specific MoAb permits an unequivocal identification of the EC (Figs 3 and 6). On the other hand, this overlapping does not allow precise quantification. Nevertheless, the presence of some monocytes and platelets in the EC subfraction is not a drawback because the use of an endothelium-specific MoAb permits an unequivocal identification of the EC (Figs 3 and 6). On the other hand, this overlapping does not allow precise quantification of the EC recovered from blood. Thus, our data were inferred from control experiments in which EC were mixed with CM (Table 1). This approach is justified by the constancy of the cellular physical properties, eg, cell specific gravity in the presence of isoosmotic gradients at constant physiologic pH.

The endothelial membrane protein recognized by the MoAb CLB-HEC 19 is also expressed when the cells are

### Table 1. HUVEC Recovery Over Percoll Gradients

<table>
<thead>
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<th>HUVEC/mL</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>Added</td>
<td>1.56 x 10^5 (SE = 2.8 x 10^4)</td>
<td>1.65 x 10^5 (SE = 6.8 x 10^4)</td>
<td></td>
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<tr>
<td>Recovered</td>
<td>1.42 x 10^6 (SE = 2.9 x 10^4)</td>
<td>1.41 x 10^6 (SE = 3.7 x 10^4)</td>
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</tr>
<tr>
<td>Recovery (%)</td>
<td>91.0</td>
<td>92.3</td>
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</table>

HUVEC recovery over a one-step gradient of 1.060 g/mL specific gravity. Cells were mixed with CM. Results of two experiments. Mean recovery is 91.6% (SE = 0.65%).

Abbreviation: n, number of counts.

Fig 5. EC number evaluation in group II patients’ blood. Bar chart of the mean number of EC per milliliter of blood in five types of blood (number of cases = 10): BVB (mean = 0.76, SE = 0.572); FVB (mean = 2.74, SE = 0.66); BAB (mean = 0.76, SE = 0.328); FAB (mean = 3.66, SE = 1.34); NVB (number of cases = 7, mean = 0.06; SE = 0.057). The two-tailed paired-sample t-test assigns no significant difference between BVB and BAB. The ANOVA shows no statistical difference between VB and AB. On the contrary, a significant difference exists between final and basal blood in both venous and arterial samples (P = .0013). The interaction between the “type” factor (VB and AB) and the “trial” factor (basal and final blood) was not significant.

Statistical comparison of the mean cell areas of immunofluorescent cells.

### Table 2. Comparison of HUVEC and PBEC Areas

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell Number</th>
<th>Mean Area (μm²)</th>
<th>SE</th>
</tr>
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<tbody>
<tr>
<td>HUVEC</td>
<td>21</td>
<td>259.76</td>
<td>23.22</td>
</tr>
<tr>
<td>PBEC</td>
<td>21</td>
<td>201.76</td>
<td>35.93</td>
</tr>
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Unpaired t Value Probability

1.356 0.1828

The two-tailed unpaired-sample t-test of the sample means shows that they are not significantly different.
suspended on glass by a cytocentrifuge (Fig 6) or held in suspension (unpublished results). This finding suggests that cell attachment and, possibly, cell viability are no prerequisites for the use of the MoAb CLB-HEC 19 as an EC marker.

The cardiac catheterization model was chosen because it is provided with a routine invasive procedure that is used under controlled and constant conditions. A recent report has suggested catheter-induced endothelial denudations in a canine model.

Our results show that the presence of EC (or remnants thereof) identified by CLB-HEC 19 after heart catheterization in both AB and VB is significantly larger (Figs 4 and 5) than in basal blood and there is no statistical difference between AB and VB. This finding suggests that the pulmonary filter may not contribute to clear the endothelial remnants present in circulation. Our data (Fig 5) also seem to suggest (even if there is no significant statistical difference) a higher BVB level of circulating EC in patients with ischemic heart disease compared with healthy subjects (NVB).

The procedure used to score group I patients' blood was much faster than the screening procedure adopted for group II. Because the screening of group II (Fig 5) confirms the results of the scoring procedure of group I patients' blood (Fig 4), the latter is recommended as a preliminary blood test. A further confirmation that the patients' blood immunofluorescent cells are indeed EC is provided by the comparison of the cell areas of randomly chosen immunofluorescent cells in two populations of cells, HUVEC and PBEC, from group II patients (Table 2), which shows no statistical difference. The cell separation and identification methods presented in this report allow an approximate 92% recovery and a detection threshold of circulating EC of about 300-fold lower than previously found. Therefore, we believe that this can be considered a promising blood test for clinical purposes.

REFERENCES

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