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RAPID COMMUNICATION

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An improved assay for enumeration of circulating endothelial cells

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Abstract Circulating endothelial cells have been established as markers of vascular disease, such as small vessel vasculitis, acute vascular rejection in renal transplant recipients, and cyclosporine-induced endothelial damage. Enumeration of these cells by immunomagnetic isolation and acridine staining remains the gold standard but necessitates considerable experience and expenditure. A simpler test would therefore be of great utility. Hence, our aim was to develop an improved simple assay to enumerate endothelial cells in peripheral blood. We had already used various surface markers to corroborate the endothelial origin of cells. Here, we studied the enumeration of cell numbers with immunomagnetic isolation and a variety of subsequent stains, such as CD31, von Willebrand's factor (vWF) immunocytochemistry, and *Ulex europaeus* lectin-1 (UEA-1). Eventually, we devised a simple protocol for enumeration using immunomagnetic isolation and a subsequent UEA-1 lectin stain. We evaluated the use of this protocol in parallel to immunomagnetic isolation and acridine counting alone in 92 renal transplant recipients who underwent renal biopsy. Recovery of various concentrations of human umbilical vein endothelial cells from blood was also studied. Immunomagnetic isolation and subsequent UEA-1 staining permits easier enumeration of circulating endothelial cells in peripheral blood. The assay is simple and easy to use, thus allowing for a more widespread use of circulating endothelial cells as a marker of vascular damage.

Keywords Circulating endothelial cells · Immunomagnetic isolation · Lectin

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Introduction

Microvascular endothelial cells have been assigned a pivotal role during the pathogenesis of inflammation and atherosclerosis. Circulating endothelial cells have recently emerged as a novel marker of vascular damage [1]. We have demonstrated that measurement of circulating endothelial cells may be of great value in antineutrophil cytoplasmic autoantibody (ANCA)-associated small vessel vasculitis [2] and renal transplantation [3, 4]. Using immunomagnetic isolation and acridine staining, however, these cells are difficult to enumerate, necessitating experienced personnel and considerable expenditure. It is particularly difficult to distinguish larger aggregates of cells and smaller cell particles from artifacts. Nonspecific binding of leukocytes to antibody-coated beads remains another matter of concern. Finally, conflicting results of various studies underline the need for standardization of criteria for identification and the test used. In this regard, a consensus conference is eagerly awaited.

We were therefore interested in developing an improved assay in order to permit a more widespread use of this marker. We had previously used various markers, including *Ulex europaeus* lectin-1 (UEA-1), to confirm the endothelial origin of cells isolated by immunomagnetic isolation [2, 4]. Here, we describe our experience with an improved protocol for the enumeration of circulating endothelial cells, using immunomagnetic isolation with a subsequent UEA-1 stain.

Materials and methods

Material

M-280 and M-450 Dynabeads were from Dynal (Norway). M-450 Dynabeads were used for counting while the smaller M-280 Dynabeads were used for studies of cell morphology since they permit better identification of the cell membrane and cytoplasm. Anti-CD 146 antibodies were from Biocytex (Marseille, France). The FcR blocking agent was from Miltenyi (Bergisch Gladbach, Germany). Rabbit antibodies against von Willebrand's factor (vWF) and fluoresceine isothiocyanate-coupled (FITC) anti-rabbit antibodies were from Boehringer (Mannheim, Germany). Sheep anti-human CD31 antibodies as well as FITC anti-sheep IgG were from the Binding Site (Heidelberg, Germany). Rhodamine-coupled UEA-

1 was from Linaris (Wertheim, Germany). The Nageotte counting chamber was from Brand (Wertheim, Germany).

Patients

To establish the technique, we first studied six patients with active vasculitis, since we had considerable experience with isolation of circulating endothelial cells in this disorder [2]. To compare our previous approach with the protocol devised here, we studied 92 renal transplant recipients who underwent renal biopsy due to a rise of their serum creatinine or as participants of our protocol biopsy scheme. Study participants and results of immunomagnetic isolation with acridine counting have been described in detail elsewhere [4].

Two-step immunocytochemistry with anti-vWF and anti-CD31 antibodies

We first studied immunocytochemistry with anti-CD31 and anti-vWF antibodies in six patients with active ANCA-associated small vessel vasculitis. Briefly, circulating endothelial cells were isolated as described elsewhere [2] using M-280 beads. Cells were fixated in 2% paraformaldehyde, washed, resuspended in buffer, and incubated with primary antibody (rabbit anti-human-vWF and sheep anti-human CD31, respectively) for 1 h. The cells were washed with buffer and incubated with secondary antibody (FITC-coupled anti-rabbit-IgG and FITC-coupled anti-sheep IgG) for 1 h. After a final washing step, cells were visualized under the fluorescence microscope.

Immunomagnetic isolation and subsequent UEA-1 stain

Anti-CD 146-coated M-450 Dynabeads were obtained as recommended by the manufacturer. Coated Dynabeads were stored at 4°C for a maximum of 4 weeks. Blood was obtained by venipuncture. We were careful to discard the first 7.5 ml blood, avoid samples from indwelling arterial and venous lines, and perform nontraumatic venipuncture. After careful rotation of the tube, 1 ml blood was mixed with 1 ml isolation buffer (phosphate-buffered saline, 0.1% bovine serum albumin, 0.1% sodium azide, and 0.6% sodium citrate) at 4°C. Twenty microliter FcR blocking agent and 50 µl antibody-coated Dynabeads (10 µg/ml) were added and mixed thoroughly. Next, the sample was mixed in a head-over-head mixer for 30 min at 4°C and washed with buffer four times inside the magnet at 4°C. Between each washing procedure, the sample was flushed ten times with buffer in a 100-µl pipette. To prevent nonspecific binding of beads to leukocytes, 100-µl UEA-1 solution (2 mg/ml) was added and incubated for 1 h in darkness. The sample was washed twice and the cell-bead suspension finally dissolved in 200 µl buffer. Cells were counted with fluorescence microscopy and a Nageotte chamber. In renal transplant recipients, immunomagnetic isolation with acridine counting alone and isolation with subsequent UEA-1 lectin stain were done in parallel. Cell numbers were compared with linear regression analysis (Pearson correlation).

Recovery

Recovery was evaluated with peripheral blood obtained from healthy volunteers. One milliliter blood was inoculated with 100, 1,000, 10,000 and 100,000 human umbilical vein endothelial cells (HUVECs). Circulating endothelial cells were enumerated and recovery calculated.

Results

Two-step immunocytochemistry with anti-CD31 and anti-vWF antibodies

CD31 immunocytochemistry yielded a clear depiction of circulating endothelial cells. The cells were oval in shape, 20–50 µm in length, and had the typical morphology of circulating endothelial cells (Fig. 1) as described elsewhere [1]. The cell surface was covered with more than four beads. Cells with more than ten beads were also seen. The signal-to-noise ratio was excellent with few artifacts. Anti-vWF immunocytochemistry yielded similar results with regard to unequivocal identification of the cells, signal-to-noise ratio, and few, if any, artifacts. Immunomagnetic isolation with subsequent CD31 or vWF immunocytochemistry took some 3 h to perform.

Immunomagnetic isolation and subsequent UEA-1 stain

The novel protocol depicted circulating endothelial cells with a size of 10–50 µm and a bead content of more than four beads per cell. This finding is in accordance with our criteria to identify circulating endothelial cells with immunomagnetic isolation and acridine staining alone [2]. With subsequent UEA-1 stain, the cells were very easily identified owing to their bright red color (Fig. 2). UEA-1-positive smaller cell particles with bead content, which are easily overlooked or confused with artifacts by immunomagnetic isolation and acridine staining alone, were also easily identified. We also identified larger aggregates of endothelial cells. A 100-µl sample in the Nageotte chamber was counted in less than 5 min due to an excellent signal-to-noise ratio (Fig. 3). There were few, if any, artifacts, such as dust or long and clearly acellular filamentous structures. These artifacts were easily identified on the basis of missing beads and/or UEA-1 negativity. The assay took 2 h to perform.

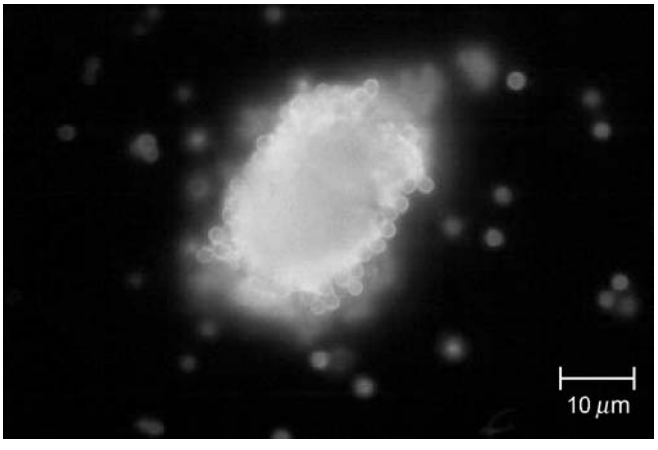

Fig. 1 Circulating endothelial cell from a patient with active vasculitis; CD31 staining (two-step immunocytochemistry)

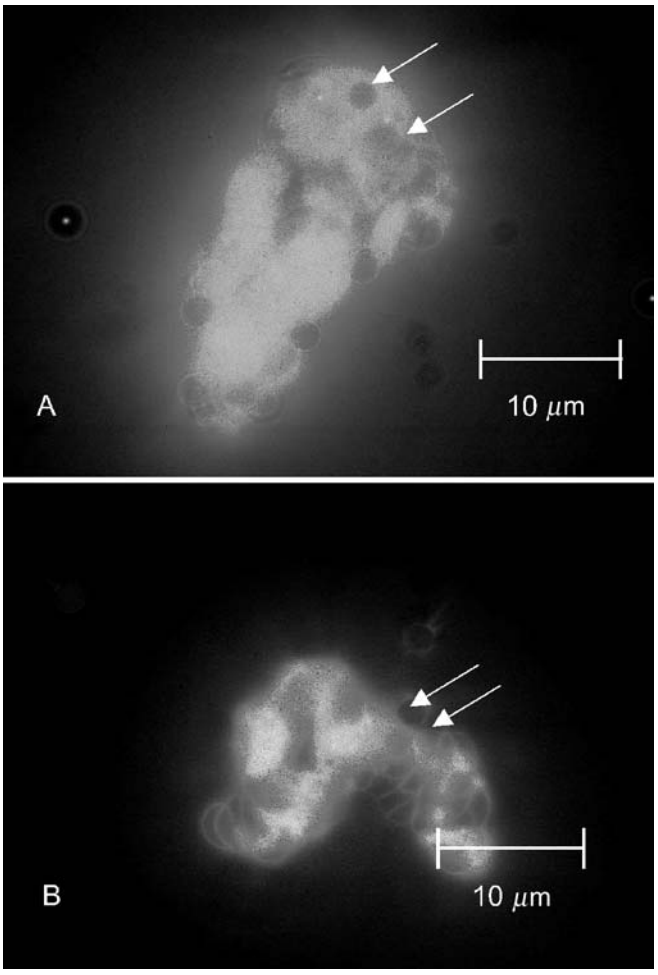

Fig. 2 Circulating endothelial cell from a renal transplant recipient with acute vascular rejection (**A**) and a patient with active vasculitis (**B**); immunomagnetic isolation and subsequent UEA-1 stain. Note that several Dynabeads (*arrows*) are attached to the surface of the cells

Correlation of immunomagnetic isolation and immunomagnetic isolation with subsequent UEA-1 lectin stain

Immunomagnetic isolation and immunomagnetic isolation with subsequent UEA-1 stain showed a highly significant and strong correlation (r=0.8219, p<0.0001, Fig. 4).

Recovery of HUVEC

Recovery of HUVECs from blood obtained from healthy volunteers was 90% with 100 cells/ml, 98% with 1,000 cells/ml, 70% with 10,000 cells/ml, and 80% with 100,000 cells/ml.

Discussion

Circulating endothelial cells were first identified in blood smears on the basis of their presumed morphology [5]. Later on, these cells were enumerated by immunocytochemistry [6]. More recently, immunomagnetic isolation has become available [1] and circulating endothelial cells

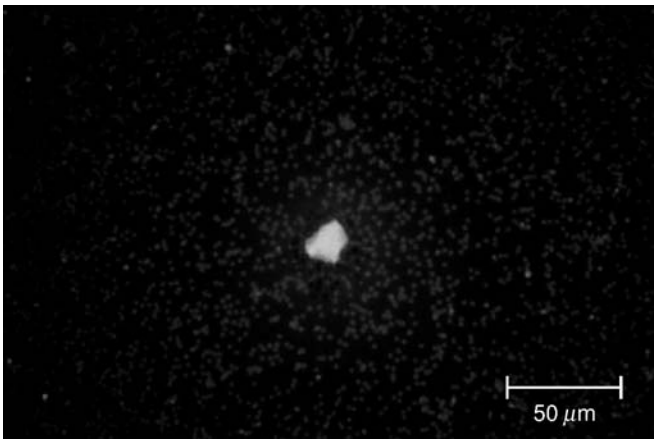

Fig. 3 Circulating endothelial cell of a patient with active vasculitis at low magnification, immunomagnetic isolation, and subsequent UEA-1 stain

have been documented in a variety of disorders [7, 8]. Notably, immunomagnetic isolation with anti-CD146 does not detect endothelial progenitor cells or pericytes (data not shown). Immunomagnetic isolation of circulating endothelial cells, however, remains cumbersome. Cell morphology may vary considerably from one disease to another and unequivocal identification of endothelial cells may hence be difficult despite the specificity of the antibody. The assay therefore necessitates considerable experience and a simpler test would be of great utility. Following our good experience with immunomagnetic isolation and acridine counting, we sought to further augment the technique by a secondary stain. In this regard, we had already used surface markers to corroborate the endothelial origin of cells yielded by immunomagnetic isolation [2, 4]. Using a conjugate-labeled anti-endothelial Dynabead is certainly also conceivable, but there is no experience with this approach at present and we therefore did not explore this possibility. The variety of markers with specificity to endothelial cells has been discussed in great detail elsewhere [9]. Endothelial markers differ markedly from one tissue to another [10] and few molecules are exclusively and uniformly expressed on endothelial cells. Several approaches were pursued. First, we tried to perform conventional immunocytochemistry with antibodies to CD31 and vWF. Several issues, however, led us to prefer a lectin stain over this approach. UEA-1 and *Bandeiraea simplicifolia* lectin-1 (BS-1) are most commonly used. UEA-1, one of the lectins of *Ulex europaeus* (gorse) has been used previously to stain endothelial cells. Notably, the ability to stain UEA-1 positive is a very stable phenomenon among endothelial cells in a variety of tissues. For example, hepatic sinusoidal endothelial cells stain UEA-1 positive while expression of vWF may be sparse or even absent [11]. Similar findings have been reported with endothelial cells in thyroid neoplasms [12]. Staining for UEA-1 has been shown with necrotic endothelial cells after stroke, confirming that the unknown epitope that binds UEA-1 is very stable. UEA-1 staining is also very specific for endothelial cells [13]. We therefore expanded our technique with a subsequent UEA-1 stain, which is easy to perform. Here, we have shown that results of the improved

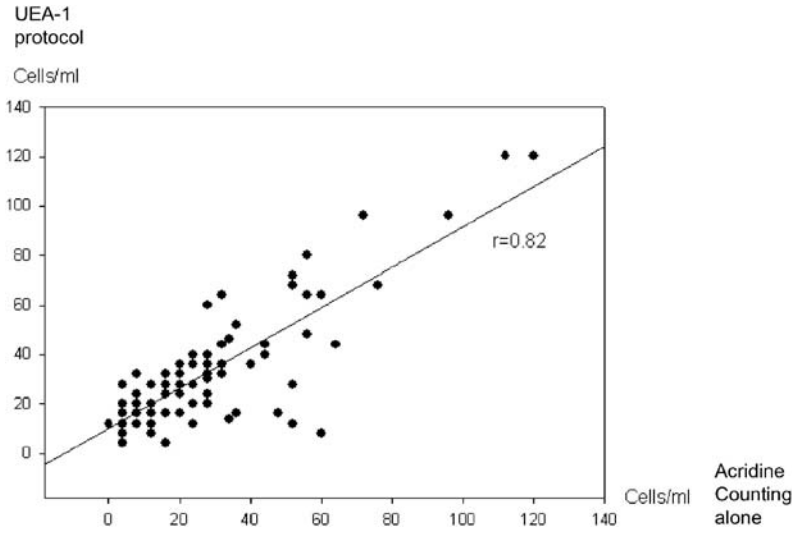

Fig. 4 Correlation of cell numbers enumerated with immunomagnetic isolation with acridine counting alone (*x*-axis) and immunomagnetic isolation with subsequent UEA-1 stain (*y*-axis)

assay correlate well with cell numbers obtained by immunomagnetic isolation alone, suggesting that both tests depict the same cells. A number of pitfalls, however, remain.

It had been shown previously that manipulation with the needle may result in dislodgement of endothelial cells at the site of the venipuncture; thus, falsely positive results may occur. We demonstrated previously that time of day and arterial vs venous sampling do not influence the number of circulating endothelial cells [2].

Nonspecific binding of beads to cells, such as granulocytes, has also been discussed elsewhere [2]. Several precautions are effective to avoid such effects, namely the use of citrate buffer, FcR blocking agent, and vigorous to-and-fro flushing of the cells during the washing procedures [2]. Recovery of HUVEC in blood was in the range of 70–98%. Notably, recovery was 90–98% with concentrations between 100 cells/ml and 1000 cells/ml, which is the range described previously in inflammatory disorders while higher concentrations do not occur in biological samples. It must be noted, however, that these results may not reflect conditions in patients, not least due to the occurrence of smaller particles with poorer recovery rates. Finally, stains with AC-133 have been uniformly negative, thus arguing against the possibility that endothelial progenitor cells are also isolated by our protocol.

The improved two-step assay described here has several advantages over immunomagnetic isolation and acridine staining alone. In comparison, the former necessitates more experience while the improved technique permits unequivocal identification, even at low magnification, of UEA-1-positive endothelial cells. Accordingly, this improved protocol may represent an important step towards consensus in this field.

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