Detection of Circulating Endothelial Cells

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Introduction

A variety of disorders affect microvascular endothelial cells[1]. From a clinical point of view, markers of ongoing endothelial injury and damage are necessary to diagnose these disorders, monitor their activity and decide about treatment. Soluble markers of endothelial damage, such as soluble von Willebrand factor and thrombomodulin have proved disappointing. More reliable markers of endothelial damage are therefore eagerly awaited.

Circulating endothelial cells were first detected in the 1970s, albeit with rather primitive techniques[2]. In essence, these cells were described in smears of peripheral blood and detection depended on the presumed morphological criteria of these cells. Later, immunocytochemistry of these cells became available[3,4]. Circulating endothelial cells, however, are rare cells when compared with other cell populations in peripheral blood. Accordingly, immunomagnetic isolation has become the gold standard for their detection and enumeration[5]. Using this methodology, circulating endothelial cells were detected in myocardial infarction[6], endothelial infection[7] and a variety of other disorders[5].

As nephrologists with an interest in vasculitis, we focused on circulating endothelial cells for two reasons. First, there is no reliable laboratory marker of disease activity in these disorders. Moreover, clues as to the pathogenesis seemed possible from the characterisation of these cells. We were also interested in the detection of these cells in renal transplant recipients since vascular disease is a major cause of morbidity and mortality in these patients. We used a protocol initially developed by Dignat-George and colleagues[7] with modifications. The antibody used targets CD 146, an antigen that is present exclusively on endothelial cells. Here, we describe our experience with immunomagnetic isolation of circulating endothelial cells in these two groups of patients and speculate on further avenues of research.

Method

Dynabeads® M-450 Pan Mouse IgG were coated with murine anti-CD 146 antibody (Biocytex, France) as recommended. Blood was obtained using venipuncture. We were careful to discard the first 7.5 ml blood, avoid samples from in-dwelling arterial and venous lines and perform non-traumatic venipuncture. After carefully rotating 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. 20 µl FCR blocking agent (commercially available) 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. Cells were stained with acridine and counted with fluorescence microscopy and a Nageotte counting chamber.

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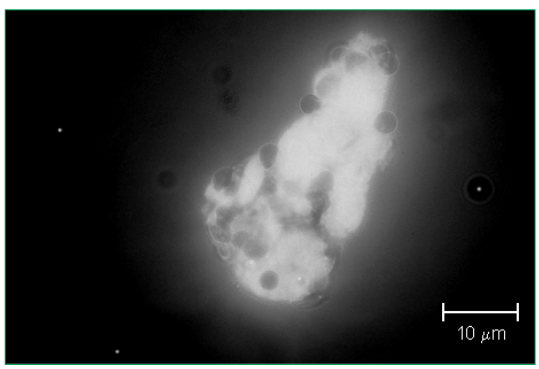

Fig. 1: Circulating endothelial cell isolated from a renal transplant recipient with acute rejection. Immunomagnetic isolation with M-280 Dynabeads®.

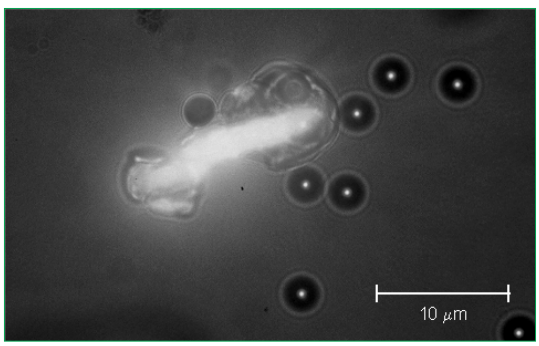

Fig. 2: Smaller endothelial cell particle isolated from a patient with vasculitis. Immunomagnetic isolation with M-280 Dynabeads®.

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Results

Using immunomagnetic isolation for three years now, we have been able to learn about the morphology of these cells and the potential pitfalls of the technique.

Initially, the main problem was non-specific binding of leukocytes to beads. We evaluated the effects of various changes in the technique by pursuing several approaches. First, we chose to stain the cells with additional markers after immunomagnetic isolation in order to know which were endothelial cells. As described elswhere[5], circulating endothelial cells may vary in their morphology from one disease to another. We have seen similar effects. While entire cells are usually in the range of 10-50-70 µm (fig. 1), larger aggregates of cells, presumably with fibrin, as well as smaller cell particles (fig. 2) were also seen. Moreover, we used Dynabeads® M-450 Pan Mouse IgG without anti-CD 146 antibody as controls. It turned out that several factors were quite effective to eliminate non-specific binding. Non-specific binding is believed to occur, at least in part, by means of the Fc receptor. Accordingly, an Fc receptor blocking agent is effective. Temperature is also an issue and working strictly at 4°C is essential. Finally, non-specific binding of cells to leukocytes is unstable while specific binding to endothelial cells is not. Therefore, vigorous flushing with a 100 µl pipette serves to further reduce non-specific binding. To ensure adequate sensitivity, recovery was studied with human umbilical vein endothelial cells. In our hands, recovery was well in excess of 90% in the range of cell numbers expected in biological specimens, i.e. 10-1000 cells/ml. By now, we are therefore quite confident with our technique and isolate circulating endothelial cells in a broad variety of clinical projects.

ANCA-asscociated vasculitis

We were able, in a preliminary study, to demonstrate that circulating endothelial cells are indeed a reliable marker of disease activity in these patients[8]. Patients with active disease have greatly elevated cell numbers when compared to healthy controls. Patients in remission have only moderately elevated cell numbers. Moreover, cell numbers declined markedly during successful immunosuppressive treatment. Using immunocytochemistry and a variety of other staining procedures, we were able to show that circulating endothelial cells are necrotic and pro-coagulant in this disorder[8]. Our findings were specific in that patients with other renal and infectious disorders, who served as disease controls, did not have elevated cell numbers. These results open up a fascinating new road towards a new and clinically useful marker of disease activity in ANCA-associated small-vessel vasculitis. We are currently exploring whether this marker is able to meet our expectations. Specifically, we would like to know whether treatment decisions can be made on the basis of cell numbers and if so in which clinical situation this might be the case.

Renal transplantation

We were able to demonstrate elevated numbers of circulating endothelial cells in renal transplant recipients[9]. In a second study, we then evaluated whether patients who receive calcineurin inhibitors as part of their immunosuppressive regimen have higher cell numbers than renal transplant recipients who do not receive these drugs. Interestingly, patients on calcineurin inhibitors had higher cell numbers than their counterparts without these drugs[10]. These findings lend further support to the hypothesis that calcineurin inhibitors damage microvascular endothelial cells.

Conclusions

Isolation of circulating endothelial cells by immunomagnetic isolation provides a fascinating means to detect ongoing endothelial damage in a clinical scenario.

The technique, however, is not without problems. Specifically, unequivocal detection of acridine-stained endothelial cells is still a matter of concern. Large aggregates of cells as well as smaller particles are often difficult to distinguish from artifacts. With considerable experience, these problems are easily overcome. Further modifications of the technique are necessary before more widespread use in a clinical scenario is possible. We are currently exploring several approaches in this regard. We are also interested in further characterisation of the cells. Therefore, we will establish double-stain immunocytochemistry, which is still hampered by the paucity of cells. Finally, interactions of circulating endothelial cells with other cell populations are conceivable. These mechanisms may play an important role in the propagation of inflammatory disease. Here, we are evaluating several models. By doing so, we may gain further insight into the pathogenesis of vascular disorders.

Ordering information

Product	Product No.	Product Volume
Dynabeads® M-450 Pan Mouse IgG	111.19	2 ml
Dynal MPC®-L	120.21	2 - 15 ml sample
Dynal MPC®-50	120.24	15 - 50 ml samples

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