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ORIGINAL ARTICLE

Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol

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Summary. *Background:* Circulating endothelial cells (CECs) have been identified as markers of vascular damage in a variety of disorders, such as myocardial infarction, vasculitis, and transplantation. CD146-driven immunomagnetic isolation has gained widespread use, but the technique is hampered by the lack of a definition of CECs and the absence of a consensus for their enumeration. *Aim:* To evaluate several variables influencing immunomagnetic isolation of CECs, formulate a definition for CECs and propose a consensus protocol for their enumeration. *Methods:* We devised a protocol based on CD146-driven immunomagnetic isolation and a subsequent confirmatory step with Ulex-Europaeus-Lectin-1 staining. In a multi-center effort, we evaluated the preanalytical and analytical phases of this protocol. We evaluated the effects of storage, anticoagulation and density centrifugation, and compiled previous experience with this technique. *Results:* Our protocol permitted unequivocal identification of CECs with acceptable reproducibility. There was an effect of storage time in that median cell numbers declined to only 87.5% of their baseline values during 24 h of storage at 4 °C. Recovery was lower with citrate than with ethylene-diamine tetra-acetic acid after 4 h of storage; density centrifugation was also associated with lower recovery. We provide a comprehensive list of technical recommendations and potential pitfalls. Finally, based on our experience with this protocol and a recent consensus workshop, we formulated a working definition for CECs. *Conclusion:* Our work represents an important step toward consensus regarding the CECs. Our recommendations represent the experience of three major centers and should now be scrutinized by others in the field.

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Introduction

Damage to endothelial cells defines a crucial step during the pathogenesis of vascular disorders. Reliable and specific markers of endothelial damage are therefore needed to gauge the extent and activity of such disorders. Soluble markers, such as von Willebrand factor (VWF), are easy to measure, but abnormal levels fail to distinguish activation from damage. Moreover, serum levels are markedly influenced by a variety of factors, such as renal failure [1]. In contrast, circulating endothelial cells (CECs) [2,3] reflect considerable damage to the endothelial cell layer. Present at a very low frequency in healthy human blood (from 1×10^{-7} to 1×10^{-5} per leukocyte, 1–20 cells mL^{-1} of venous blood) [4], CECs are defined by morphological features and expression of endothelial markers, such as VWF and CD146, a key marker in the identification of CECs [5,6]. Enumeration of these cells, however, necessitated expenditure and experience [7] until more elegant techniques for the detection of these cells became available [4]. In this regard, immunomagnetic isolation, used for the first time in 1992 [8], has been employed for the last decade. The technique depends on the use of paramagnetic particles coated with antibodies directed against the CD146 molecule (mainly S-ENDO1 [8] and P1H12 [2] antibodies). Unfortunately, the technique is still cumbersome and necessitates experience to identify CECs on the basis of morphology and the number of beads attached. In addition, activated T cells may also express CD146. We have therefore modified the CD146-based immunomagnetic separation protocol based on the use of an additional stain with Ulex Europaeus Lectin-1 (UEA-1), a specific marker of endothelial cells [9]. Another issue that remains unresolved is the lack of consensus regarding identification of CECs and their discrimination from endothelial progenitor cells.

Here, we therefore propose a step toward consensus for the enumeration of CECs. We describe a consensus protocol as well as preanalytic and analytic steps to ensure reliable detection and enumeration of CECs. Finally, we propose a definition of these cells.

Methods

Standard protocol

1. Anti-CD 146-coated M-450 Dynabeads (Dynal, Norway) are prepared as recommended by the manufacturer [10] and stored at 4 °C for a maximum of 4 weeks.
2. A sample of peripheral blood is obtained with non-traumatic venepuncture (use of tourniquet, selection of a large vein, and avoidance of needle movements). Two 7, 5 mL ethylene-diamine tetra-acetic acid (EDTA) tubes are filled and the first tube is discarded. One milliliter of blood from the second tube is mixed with 1 mL buffer (phosphate-buffered saline, PBS), 0.1% bovine serum albumin, 0.1% sodium azide, and 0.6% sodium citrate) at 4 °C. Twenty microliters of FcR-blocking agent (Miltenyi, Bergisch Gladbach, Germany) and 50 μL antibody-coated Dynabeads (10 μg mL^{-1}) are added and mixed thoroughly.
3. The sample is mixed in a head-over-head mixer for 30 min at 4 °C and washed with buffer four times inside the magnet (Dynal MPC-L, Dynal, Norway) at 4 °C. Between each washing procedure, the sample is flushed 10 times with buffer in a 100 μL pipette.
4. 100 microliters of a 2 mg mL^{-1} FITC-coupled UEA-1 solution (Sigma-Aldrich, St Louis, MO, USA) are added and incubated for 1 h in darkness. The sample is then washed twice and the cell-bead suspension finally dissolved in 200 μL buffer. Cells are counted with fluorescence microscopy at 553 nm and a Nageotte chamber (Hecht, Sondheim, Germany).

Effects of storage on cell numbers

To assess the effects of storage on cell numbers across different ranges of cell numbers (i.e. from around 20 cells mL^{-1} to around 500 cells mL^{-1}), we studied 10 patients with various disorders (three renal transplant recipients postoperatively, two lung transplant recipients, one heart transplant recipient, three patients with active systemic vasculitis, and one stable patient on dialysis). Samples of whole blood were split into eight aliquots at baseline. CECs were then enumerated with standard protocol at baseline and after 2, 4, and 24 h storage at room temperature and at 4 °C, respectively.

Effects of anticoagulation on cell numbers

Human umbilical vein endothelial cells (HUVEC) were isolated with standard methodology [11]. Two milliliters of blood from six healthy volunteers (healthy members of laboratory staff who were non-smokers and on no medication) was sampled in tubes containing either 1.6 g L^{-1} EDTA or 0.129 mol L^{-1} sodium citrate. Samples of blood were then spiked with HUVEC to achieve final concentrations between 20 and 50 cells mL^{-1}. Endothelial cells were isolated from whole blood by standard protocol. Isolation was performed immediately and after 4 h of storage at room temperature. Correlation between cell numbers in EDTA- and citrate-based samples was calculated. Cell numbers after 4 h were calculated as percentage of baseline numbers.

Effect of density centrifugation

We tested the hypothesis that there is no difference in the number of CECs in the mononuclear cell fraction of a density centrifugation compared with venous blood. To do this, we collected two 4 mL vacutainers of venous blood from 12 healthy subjects and 12 patients admitted to the coronary care unit with an acute coronary syndrome. One vacutainer was prepared according to our standard procedure (see above). Blood from the second vacutainer was removed to a 15 mL conical plastic tube and an equal volume of PBS was added. This diluted whole blood was gently layered onto 6 mL Histopaque (Sigma, Poole, UK, density 1.077 g mL^{-1}) and was centrifuged at 400 *g* for 30 min at room temperature. The mononuclear cell interface was carefully pipetted off and was washed twice in PBS by centrifugation at 1000 *g* for 20 min and brought to a volume of 4 mL in PBS (i.e. the same volume as a vacutainer of venous blood). CECs were then prepared from both venous blood and the purified mononuclear cells according to the standard techniques (i.e. starting with an addition of anti-CD146-coated immunobeads).

Number of beads per CEC

To obtain data on the frequency of Dynabeads™ per cell, we counted the number of beads on 200 well-defined preparations of CECs from five healthy controls (healthy members of laboratory staff who were non-smokers and on no medication) and from 20 patients with cardiovascular disease (acute coronary syndrome, stroke, atrial fibrillation, etc.).

Reproducibility

Reproducibility was tested with HUVEC-spiked samples of peripheral blood obtained from 11 healthy subjects. Samples of peripheral blood were collected in EDTA and HUVEC were isolated with standard methodology. Samples of peripheral blood were spiked with HUVEC to achieve concentrations between 10 and 100 cells mL^{-1}. Three to four replicates were performed for each sample and coefficient of variation (CV) was calculated.

Problems and recommendations

We reviewed our experience published previously [3,4,12–21] and included the data presented here. Based on these data and

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our own experience, we compiled a list of problems with the isolation and enumeration of CECs and provide recommendations. Those recommendations were graded (experimental data vs. opinion).

Statistics

Data were subjected to normality tests (Shapiro–Wilks) to determine the distribution. Data distributed normally are presented as mean (SD), non-normally distributed data are presented as median (full range). Data were correlated by Spearman or Pearson's methods. Paired data were analyzed by paired *t*-test or Wilcoxon's test. Results for the experiment comparing the standard method with the method using cells purified by density centrifugation are also shown as a Bland–Altman plot.

Results

Effects of storage on cell numbers

Figures 1 and 2 display cell numbers during storage at room temperature and 4 °C, respectively. In these figures, samples were grouped into the patients with the lowest five cell numbers (Fig. 1A) and those with the five highest cell numbers (Fig. 1B), respectively, solely to permit better visualization of the effects. At baseline, cell numbers were 36–472 cells mL^{-1} (median 64 cells mL^{-1}). At room temperature (Fig. 1), cell numbers were 12–412 cells mL^{-1} (median 46 cells mL^{-1}) after 2 h, 16–224 cells mL^{-1} (median 36 cells mL^{-1}) after 4 h and 4–64 cells mL^{-1} (median 16 cells mL^{-1}) after 24 h. At 4 °C (Fig. 2), cell numbers were 36–460 cells mL^{-1} (median 66 cells mL^{-1}) after 2 h, 28–340 cells mL^{-1} (median 56 cells mL^{-1}) after 4 h and 24–312 cells mL^{-1} (median 56 cells mL^{-1}) after 24 h. Hence, at room temperature, median cell numbers declined to 25% of their baseline value while at 4 °C median cell numbers declined to only 87.5% of their baseline values during 24 h of storage.

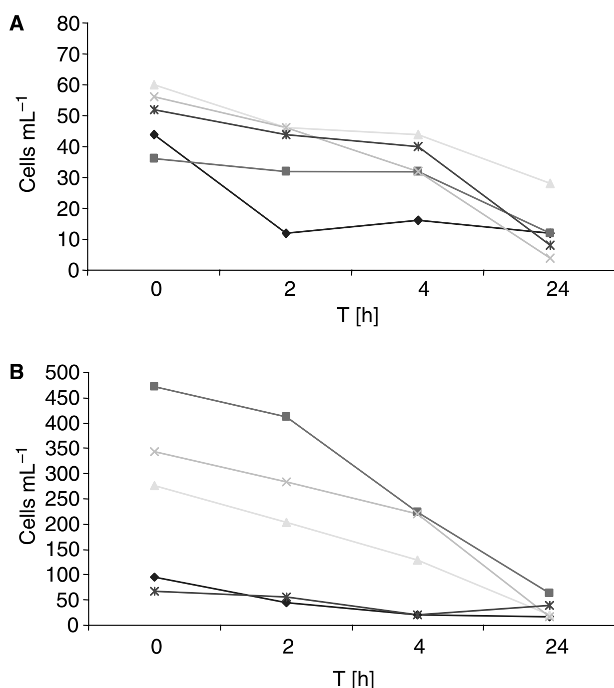

Fig. 1. Effect of storage on cell numbers at room temperature; (A), five patients with low cell numbers. (B), five patients with high cell numbers.

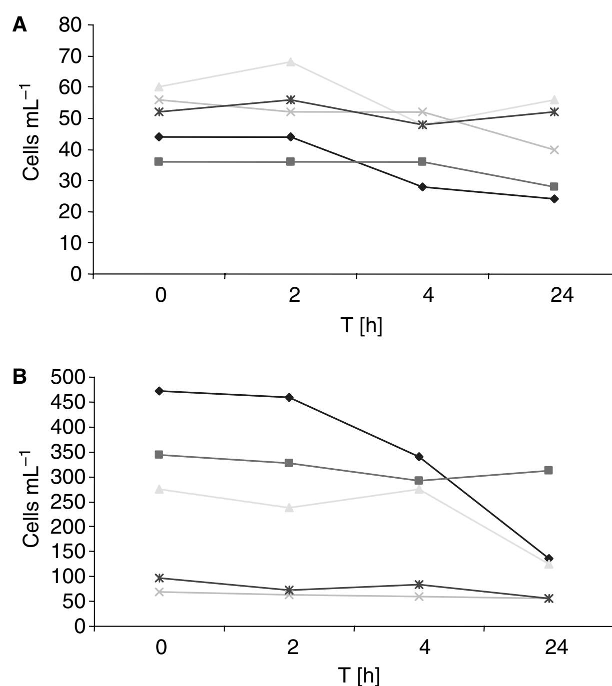

Fig. 2. Effect of storage on cell numbers at 4 °C. Panel A, five patients with low cell numbers. Panel B, five patients with high cell numbers.

Effects of anticoagulation on cell numbers

A strong and significant correlation was found between baseline cell numbers enumerated in EDTA- and citrate-based samples ($r^2 = 0.99$, $P = 0.0028$). After 4 h, however, cell numbers correlated much less ($r^2 = 0.35$, $P = 0.0583$). Average recovery with EDTA was 79.3% (range 43.3–105.9%) while average recovery with sodium citrate was 34.8% (range 6.5–46.7%).

Effect of density centrifugation

In the blood from 12 patients with an acute coronary syndrome, median CEC numbers were 4.25 (range 1–23.5) cells mL^{-1} using the standard methods, but were 3.37 (range 1–10.7) cells mL^{-1} using the mononuclear cells prepared by density centrifugation ($P = 0.026$, Wilcoxon's text). In healthy controls, CECs were 1.37 cells mL^{-1} (range 0–4) using the standard technique and 1.00 cells mL^{-1} (0.5–3) using the mononuclear cell preparation ($P = 0.919$). The median difference in CEC counts between the two methods was

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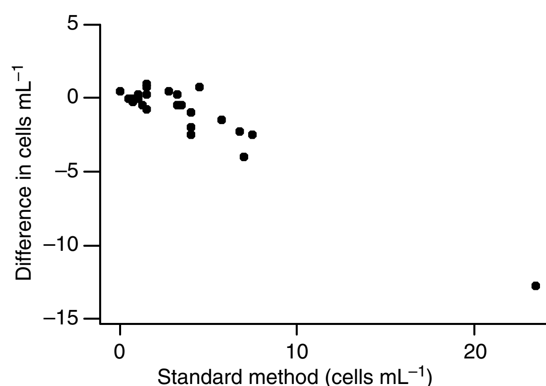

Fig. 3. The figure refers to the absolute difference in the result between the two methods when plotted in terms of the result using the standard method. Note approximate inverse correlation ($r = -0.95$, $P < 0.001$) meaning that the difference between the methods is greatest when cell numbers are high.

1.75 cells mL^{-1} in acute coronary syndrome patients, and was 0 cells mL^{-1} in healthy controls ($P = 0.464$, Mann–Whitney *U*-test). Bland–Altman plots are shown in Fig. 3. These indicate an increasing difference between the two methods as the number of CECs per milliliter increases. Notably, almost all data points are below the line of equivalence indicating lower numbers in the density centrifugation method.

Number of beads per CEC

Numbers of beads per cell from 200 separate CECs preparations are shown in Fig. 4. The distribution of beads rosetting to a single CEC is normal (Shapiro–Wilks test). The mean number (SD) was 14 (3.6) beads per cell. We took 20 beads per cell as an arbitrary cut off point because the crowding effect made accurate counts impossible. There were 13 events of > 20 beads per cell in these 200 preparations, all of which we took to be greater then one cell (i.e. a clump or sheet). None of these multi-cell events was present in the healthy controls.

Reproducibility

There was a marked difference in the CV between samples with low and those with high cell numbers. For low cell numbers

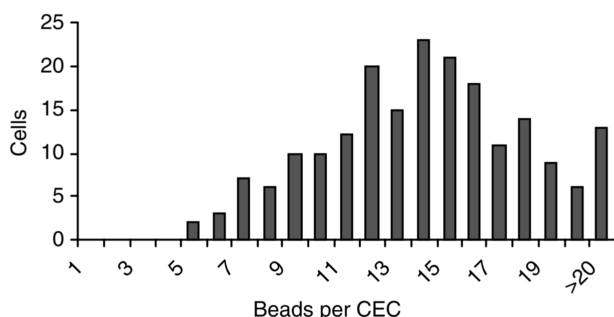

Fig. 4. Frequency distribution of the number of Dynabeads per CEC.

(mean < 20 cells mL^{-1}), CV varied from 8% to 44% (mean 28.0, SD 16.7). For high cell numbers (mean > 30 cells mL^{-1}), CV varied from 4% to 25% (mean = 13.7, SD 9.0).

Problems and recommendations

Table 1 lists all problems encountered with immunomagnetic isolation and recommendations to circumvent them.

Discussion

The concept of CECs as markers of vascular damage is not new. Initially, cells were identified on the basis of their presumed morphology in smears of peripheral blood. Since then, a number of techniques have been introduced to facilitate isolation and enumeration of these cells [4]. In this regard, CD146-driven immunomagnetic isolation has become the most commonly used method. Other investigators have used direct antibody staining (often cytospin preparation) for CD146 or other antigens without an enrichment step, some have used flow cytometry [22,23].

CD 146 is a trans-membrane glycoprotein, which is constitutively expressed on endothelial cells [24], pericytes [25] trophoblasts, mesenchymal stem cells, malignant tissues, and a subpopulation of activated T cells. Therefore, CD146-based magnetic separation rarely provides an absolutely pure fraction of endothelial cells and the procedure should be accompanied by a second step (generally fluorescence microscopy) to confirm that all separated cells are of endothelial origin and provide a more precise quantitation. Although enumeration of CEC is not a standardized procedure, there is a good degree of agreement between laboratories with CD146-driven immunomagnetic isolation and most normal individuals have values in the order of < 10 cells mL^{-1}. Using CD 146-driven immunomagnetic isolation, a broad variety of disorders have been studied [4] and clinically meaningful information has been obtained [16]. Elevated cell numbers are a reliable and sensitive marker of ongoing vascular damage and cell numbers correlate with disease activity [16].

We first addressed some preanalytical variables. Several methodological issues have been dealt with previously. We have shown elsewhere that there is no difference between arterial and venous samples [16]. Likewise, we have excluded diurnal changes [16]. We have also demonstrated an effect of venepuncture in that traumatic procedures may dislodge endothelial cells from the vessel wall [26]. Hence, we recommended discarding the first tube of blood [16]. Whether or not blood can be obtained from indwelling catheters remains unknown but dislodging of endothelial cells by these catheters is conceivable. Pending further experimental data in this matter, we therefore discourage the use of samples from indwelling catheters.

We present data regarding the effects of storage and anticoagulant. On the basis of our data, we recommend storage of samples at 4 °C as soon as possible and not to be stored longer than 4 h. Mail transfer of samples at 4 °C seems to be acceptable with losses in median cell numbers of 12.5% during 24 h. These

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Table 1 Potential pitfalls in isolating and counting of CECs with recommendations on how to avoid them

Problem	Recommendation	Evidence level (Ref. [#])
Preanalytical steps		
Site of sampling		
Arterial vs. venous	No effect	Experimental data [8,16]
Use of indwelling catheters	Discouraged, perform separate venepuncture	No data/opinion
False positives because of traumatic venepuncture and dislodgement of endothelial cells from vessel wall	Discard first tube of blood, aim to perform non-traumatic venepuncture (use of tourniquet is acceptable; select large vein, avoid needle movements)	Experimental data [8,16,26]
Diurnal variations in circulating endothelial cells (CEC) numbers	No effect	Experimental data [16]
Sample processing	Use whole blood (not Ficoll or other leukoconcentrate)	Data presented in this paper
Effect of storage time and temperature	Store at 4 °C, storage time should be < 4 h; overnight mail transfer at 4 °C may be acceptable although with some losses in cell numbers (12.5% in our study)	Experimental data presented in this paper
Effect of hemolysis, excessive hyperlipidemia	No data	No data
Anticoagulation	Use ethylene-diamine tetra-acetic acid (EDTA) (sodium citrate discouraged) Heparin: no data	Experimental data presented in this paper
Analytical steps		
Definition of CEC	A nucleated or a nuclear cell that exceeds 10 μm in length that has more than five immunomagnetic beads attached and stains Ulex Europaeus Lectin-1 (UEA-1) positive	Experimental data [3,9,16] and in this paper (number of Dynabeads per cell)/opinion (size)
Specificity	Ensured by CD146 and lectin stain Non-specific binding of leukocytes reduced by using an Fc-blocking reagent	Experimental data [16,19]
Sensitivity and range of the assay	Between 1 cell mL^{-1} and 1000 cells mL^{-1} (higher cell numbers very rare in human samples)	No data/opinion
Reproducibility	Perform one replicate if low cell number has been obtained and measurement may influence clinical decision making	Data presented in this paper
Sample stability (between isolation and counting)	Aim for immediate counting after enumeration	No data/opinion
Influence of isolation on cell morphology	Consider influence	Apoptosis and necrosis are not induced by the isolation procedure [16] although more subtle changes cannot be ruled out

findings are of considerable importance as we have previously described the use of CECs in vasculitis, a disorder that is rare and often treated by specialists far away from the patient's home. It must be emphasized that we have only studied recovery after storage at rest. Recovery may be even lower if tubes sustain repetitive movements during mail transfer.

Regarding the use of anticoagulant, we have shown that use of sodium citrate and EDTA leads to comparable results if the isolation procedure is performed immediately. We did not evaluate the effects of heparin. After 4 h of storage, however, recovery of HUVEC was lower with citrate than with EDTA. We presume that these findings with HUVEC can be transferred to clinical samples. As some form of storage is often impossible to avoid, we recommend EDTA.

Several investigators [22,27–30] have used density centrifugation to prepare CECs. Furthermore, there is wide variance in the precise method used (e.g. centrifugation for 25 or 30 min at 250–2000 *g*). Although there was no difference in CECs measured using the mononuclear cells prepared by density centrifugation compared with whole venous blood in healthy controls, numbers were about 20% lower in patient's blood. We therefore suggest that use of mononuclear cells prepared by density centrifugation to isolate CECs may lead to loss of an important sub-fraction. A summary of recommended preanalytical conditions and its evidence is provided in Table 1.

Regarding the analytical step, CD146-driven immunomagnetic identification of CEC is fraught with a number of pitfalls. Firstly, the technique is cumbersome and identification of cells requires time and experience. In essence, identification of cells is achieved on the basis of cell morphology/size and bead content. Both factors, however, are extremely variable and subjective. Moreover, cell morphology differs between disorders. We have therefore developed a modified protocol of immunomagnetic isolation using subsequent lectin staining with UEA-1 as a confirmatory step [9]. This protocol greatly facilitates identification and enumeration by virtue of its additional specificity.

We did not correlate our data with flow cytometry, as that technique has provided markedly different results. In theory,

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immunomagnetic isolation should be more sensitive, given the fact that this approach was originally invented for the detection of rare events in peripheral blood. However, flow cytometry has detected much higher cell numbers of CECs in healthy volunteers [23]. At present, this issue must be regarded as unresolved [14].

The specificity of CD146 for differentiated cells of the endothelial lineage is beyond doubt. What remains unclear is whether and to what degree our technique may isolate endothelial progenitor cells. In our hands, the protocol described here isolates a distinct cell population that does not stain with CD 133, a classical progenitor cell marker [19]. Moreover, the size of the CEC population often exceeds 10 μm, which is not compatible with the typical size of endothelial progenitor cells. Finally, the morphology of our cells indicates considerable damage or even necrosis. These findings, we believe, are not in keeping with the assumption that they might be progenitor cells.

We have shown that the reproducibility of cell numbers is acceptable in samples with high cell numbers. Reproducibility of cell numbers is less reliable in samples with low cell numbers. This observation derives from the fact that cells are counted in the counting chamber and multiplied by a factor that depends on the characteristics of the counting chamber. Thus, inter-observer discrepancies are more relevant in low cell numbers. We therefore recommend replicate assays if low cell numbers are obtained and clinical decisions depend on CEC measurement (e.g. the exclusion of active vasculitis or change in immunosuppression). A summary of recommended analytical conditions and its evidence is also provided in Table 1.

Another major obstacle is the lack of a consensus definition for a CEC. The lack of a single marker for differentiated endothelial cells necessitates a combination of different criteria and markers. Based on our experience, we propose the following definition: a CEC is a nucleated or anuclear cell that exceeds 10 μm in size and has more than 5 immunomagnetic beads attached. The rosetted cell stains positive with at least two endothelial markers (for example CD146 and UEA-1) and is negative for leukocyte markers (e.g. CD14 and CD45). Moreover, in contrast to circulating endothelial progenitors, a CEC is negative for CD133 and cannot give rise to colonies with a high proliferative potential.

Conclusion

Our work represents a step toward consensus regarding isolation and enumeration of CECs. Our recommendations expressed here represent the experience of three major centers and should now be scrutinized by others in the field.

Conflict of interest

Alexander Woywodt gave a talk at a symposium for Dynal, the manufacturer of Dynabeads in April 2001, and received honorarium. The authors declare no further conflict of interest.

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