

Editorial Focus

Immunomagnetic isolation and FACS – competing techniques for the enumeration of circulating endothelial cells

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The concept of circulating endothelial cells (CEC) as markers of vascular damage is not new. Some 30 years ago, CEC were already observed in peripheral blood (1), to serve as indicator for vascular injury. Other markers include thrombomodulin, von Willebrand factor, E-selectin, and endothelial microparticles (2). Since the early observations of CEC in smears of peripheral blood, several advances have been made in detecting and characterizing these cells. While CEC can be defined by indirect immunofluorescence with antibodies to von Willebrand factor (3), in 1991, two groups reported monoclonal antibodies to a new surface antigen on endothelial cells and used them to isolate CEC (4, 5). This antigen CD146 was subsequently characterised as adhesion molecule Mel-CAM (6), involved in cell-cell cohesion (7) and actin cytoskeleton rearrangement (8). During the last 15 years, CD146 driven immunomagnetic isolation has become the predominant approach to isolate CEC under numerous conditions (9, 10). The clinical use of CEC has been advocated (11–13), but several obstacles hamper their isolation. Most importantly, CD146 mediated immunomagnetic cell isolation lacked consensus. But even the definition of a CEC remained elusive during the past 15 years, although an ever-increasing number of studies employed this marker. Cell size is of particular importance, since CEC must be distinguished from endothelial microparticles. To make matters more complicated, CEC appear in very different phenotypes, such as anuclear carcasses, fragments or even multi-nucleated conglomerates (9). Here, immunomagnetic isolation has the advantage of actually “seeing” the cells.

As with any other diagnostic marker, consensus is essential to compare measurements between various laboratories. In 2004, the need for consensus was formulated at a workshop during the International Society on Thrombosis and Haemostasis meeting in Ljubljana, Slovenia (10). Eventually, a multi-centre project was initiated. As a first step towards consensus, CD146 driven immunomagnetic isolation was taken one step further including an additional lectin stain (14) and agreeing with all participating centres on a new CEC isolation consensus protocol, containing definition of CEC characteristics (15) that should

now be scrutinized by others in the field (see <http://www.eurocec.com>).

Other problems of CD146-driven immunomagnetic CEC isolation remain since recent studies suggest that the technique may also capture endothelial progenitor cells (EPC) (16). Unfortunately, EPC themselves remain ill-defined as to phenotype and surface markers, and using *Ulex europaeus* agglutinin 1 (UEA-1) as an additional marker does not exclude EPC. Some clinicians may find this flaw excusable, provided that the cell count mirrors vascular damage. We certainly disagree. Measuring CEC and EPC separately is worthwhile since EPC may reveal very different information about the severity and time course of vascular damage. EPC are also influenced by a great variety of other factors, such as drugs (17). A composite marker of CEC and EPC is therefore critical and the protocol for immunomagnetic isolation should be modified to avoid co-isolation of EPC.

For some time now, fluorescence-activated cell sorting (FACS) has been applied to isolate CEC, and this approach has some promise to offer, particularly when EPC must be excluded. Specifically, multi-parametric studies should be able to overcome this problem. Moreover, FACS approaches are less time consuming, more affordable for diagnostics and easily amenable to standardization once consensus has been reached. So far, no studies have compared CD146-driven immunomagnetic isolation and FACS of the same CEC-preparation. In this issue of *Thrombosis and Haemostasis*, Goon et al. (see pages 45-52) report a direct comparison of the two approaches in three different groups of patients and healthy controls (18). They observed reasonable parameter agreement in the medium range in patients but less correlation at higher and lower CEC numbers. Interestingly, they also detected less correlation in healthy controls. Notably, FACS studies report enumeration of CEC in healthy controls that differ by several orders of magnitude from immunomagnetic isolation of CEC (10), the latter reaching cell numbers in the range of 0–10 cells/ml. Using immunomagnetic cell isolation, CEC in healthy controls amount to between 0.01% to 0.0001% of all mononuclear cells. In our experience with vas-

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culitis (12), patients in remission, granulomatous disease or early relapse, often have rather low cell numbers (19). A technique that is associated with problems in low cell numbers would be fraught with problems in this clinical scenario. Goon et al. also observed less correlation between FACS and immunomagnetic isolation at higher cell numbers and speculate about possible explanations. From a clinical point of view, discordance in higher cell numbers may not matter too much. In highly active disease, such as systemic vasculitis, the symptoms may be impressive enough so that the CEC count merely confirms a clinical diagnosis.

Another issue that needs consideration is the choice of surface markers in FACS studies of CEC. Goon et al. only counted CD34/CD146 double positive cells, and great care was taken to exclude leukocytes. It must be noted that other FACS studies of CEC have used different markers, such as CD31 and CD105. In particular, the issue of consensus is not limited to immunomagnetic isolation. At present, there is no attempt to reach a consensus protocol for FACS isolation of CEC (20, 21) and to avoid the

inconsistent values and inter-individual differences between CEC numbers in healthy probands.

Finally, the study by Goon et al. is reassuring that immunomagnetic isolation and FACS of CEC do not yield completely different cell numbers. At present, both techniques remain in close "competition" and it is likely that consensus on FACS isolation of CEC is challenged by the need for improved sensitivity in low and very low cell numbers. Immunomagnetic isolation may have to face yet another technical level to exclude EPC, but then it may become too elaborate, time-consuming and expensive for clinical studies. It will be interesting to see in which way both techniques may evolve and gain reliability in patients' diagnostics.

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