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Depletion of CD45 as Pre-Enrichment Step for Rare Cells Detection Using a Novel Immunomagnetic Technology

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Abstract

Isolation of rare cells from peripheral blood may be helpful either in the detection of circulating tumor cells and directing aggressiveness of therapy, or it can be helpful for prenatal diagnosis by the enrichment of fetal cells from maternal blood. The major limit of detection rare cells using magnetic beads is primarily influenced by the non-specific binding of non-target cells. The depletion of non-target cells is considered an important prerequisite for rare cells detection.

In this study, we examined and compared the depletion efficiency of CD45 cell from peripheral blood samples using two different forms of immunomagnetic separation technology namely: the Cell enrichment process (CEPir) and DynaMAG technology.

Our results indicate significantly lower number of CD45 cells in negative fractions using the CEPir technology. Moreover, the CD45 depletion efficiency was found to be higher in CEPir technology when compared with the DynaMAG.

Negative selection is a promising approach for isolation of rare cells, we concluded that the negative selection (depletion) of leukocytes using CD45 marker is an effective pre-enrichment step for the detection of rare cells using CEPir technology. Further studies are required to validate its efficacy at capturing specific rare cells for downstream application.

Keywords: Rare cells, Immunomagnetic, CD45 depletion

1. Introduction

Isolation of rare cells from peripheral blood may be helpful in determining prognosis (Redding, 1983); (Mansi, 1987); (Cote, 1991); (Mansi, 1991); (Ellis, 1989) genetic diagnostic (Ganshirt-Ahlert, 1993); (Yamanishi, 2002); (Guetta, 2004) and directing aggressiveness of therapy. For an example, circulating tumor cells (CTC) are rare cells that circulate into the blood stream of cancer patients (Allard, 2004); (Racila, 1998). The isolation and detection of CTC is essential to understand the relationship between cancer metastasis and CTCs (Fiegl, 2002). In addition, detection of CTC is a main key of directing cancer therapy. However, new technologies to detect CTC faced many technical limitations (Baccelli, 2013).

CTCs are the earliest hallmark of tumor invasion. They are known to circulate in the blood for months or years before metastases develop (Paterlini-Brechot, 2007); (CA, 2009); (Rhim, 2012) and can thus reliably help identify patients in the intermediate stage between localized and metastatic, who are presently not identified and thus either untreated or treated with non-personalized protocols.

Breast, prostate and colorectal cancers of epithelial origin carry a shared cell surface marker known as Epithelial Cell Adhesion Molecule or EpCAM. Immunomagnetic antibodies against EpCAM have been used to target the CTCs, followed by magnetic separation and optical analysis to isolate and reliably detect CTCs (CellSearch® Circulating Tumor Cell Test, Janssen Diagnostics, LLC, Raritan, NJ) (Riethdorf, 2007); (Tibbe, 1999). This method of using an antigen expressed by the tumor cells as means of their capture and isolation is referred to as "positive selection". However, most circulating melanoma cells (CMCs) do not express EpCAM. The non-specific binding of antibodies to non-target cells constitutes a major limitation in the detection and isolation of rare cells from peripheral blood; therefore, pre-enrichment steps are needed to increase isolation specification.

An alternative to the "positive selection" CTC capture strategy is "negative selection" in which cells of interest are enriched by depletion of unwanted cells. Negative selection is advantageous for separating cells with poorly characterized immunophenotype. Importantly, the enriched cells are "untouched" by the labeling ligands, and therefore, less likely to be activated.

The depletion process consists of removal of most normal white blood cells by magnetic separation and the unwanted cells in this case are labeled with the universal marker CD45 that is highly expressed on all white cells membrane.

In this present study, we compared two immune-magnetic separation technology for CD45 depletion from peripheral blood, the DynaMAG-15 (Life Technology, Carlsbad, CA, USA) and CEPir device (BioCEP Ltd, Yokneam Illit, Israel) for efficiency of depletion, cell purity and yield.

2. Materials and Methods

2.1 Peripheral Blood Sample

15-20 ml Blood samples were obtained from healthy adult volunteers with Helsinki approved

protocol (N = 10). Samples were collected in vacutainer tubes containing EDTA as anticoagulant and transferred into a 50 ml tube.

The whole blood was diluted in ratio of 1:2 with buffer (PBS that includes 0.1% BSA and 2 mM EDTA). The blood samples were centrifuged at 600 g (Beckman Coulter, Inc.) for 10 minutes at 22° C. The plasma was carefully aspirated out and discarded after centrifugation. The blood was then suspended to the original volume with the same buffer.

2.2 Anti-CD45 Antibody and Immunomagnetic Labeling

The blood sample was split into three tubes, with each tube containing 5 ml of blood. Two tubes were labeled by immunomagnetic labeling for two different separation techniques while one tube was reserved for cell count. 500 µL of Dynabeads CD45 (Life Technology, Carlsbad, CA, USA) was added to 5 ml of washed whole blood and incubated for 30 minutes.

2.3 Depletion of Leukocytes by DynaMAG and CEPir

The DynaMAG static magnet and CEPir device were compared for CD45 depletion efficiency from whole blood samples. For the DynaMAG separations, 5 ml labeled whole blood samples with Dynabeads CD45 were placed into the DynaMAG for 2 minutes. The supernatant was then transferred to a new tube and the sample was washed with 1 ml fresh buffer. The washing process was repeated three times and the new tube with the supernatant "negative fraction" was analyzed for comparison

For CEPir separations, 5 ml labeled whole blood samples with Dynabeads CD45 were placed into the CEPir device (Figure 1). CEPir is a semi-automatic device; the separation process is less than ten minutes and contains four main stages:

a) Priming: Automatically filling of the CEPir tubes with fresh buffer so that the cells are carried and supported by the liquid medium and are not in friction with the walls of the tube.

b) Separation: The sample was loaded manually into the sample vessel, while the two electromagnetic were turned on. The sample flowed through the four separation areas and the labeled cells were drawn to the magnetic field while the unlabeled cells flowed to the negative fraction.

c) Rinse: Additional rinsing with buffer was done when the magnets were still on, to wash the unlabeled cells to the negative fraction.

d) Collection: The magnets were turned off; fresh buffer was flown in order to collect the labeled cells to the positive fraction. The "negative fraction" was then analyzed for comparison with DynaMAG.

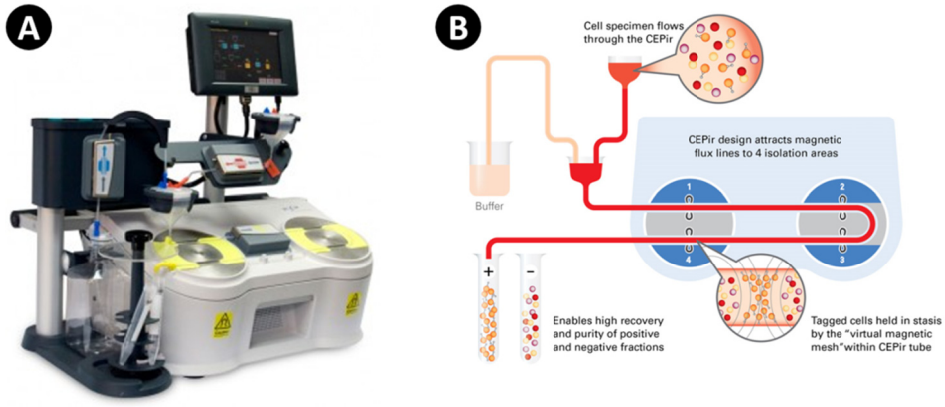

Figure 1. CEPir device and the technology principle. The CEPir's flow through technology and novel design (A). As the blood sample flows through the CEPir, labeled cells are held in stasis within the virtual mesh in 4 isolation areas. The electromagnets create strong magnetic fields that enable high isolation. The proprietary software allows creation of new protocols (B)

2.4 Flow Cytometry Analysis of the Negative Fractions in the DynaMAG and CEPir

The negative fractions were centrifuged 300g for 10 min, re-suspended in 100 µl buffer (PBS that includes 0.1% BSA and 2 mM EDTA). The cell pellets were then incubated with 10 µl of the anti CD45 (PC5)-labeled monoclonal mouse anti-human antibody (Beckman Coulter, Marseille, France) for 10 min at 4^0C. The cells were washed, centrifuged and re-suspended in 0.5 ml of buffer for flow cytometry analysis. The measurements were carried out with a Flow Cytometry (MACSQuant Analyzers, Miltenyi Biotec, Germany). The control cells were only incubated with the anti CD45 (PC5) for cell count and reference. For data evaluation, MACSQuant software was used.

3. Results

The depletion of CD45 is important for many rare cells isolation and is used as a pre-enrichment step to deplete unwanted cells and avoid non-specific isolation. Dynabeads CD45 from life technology was used for leukocyte depletion from fresh peripheral blood sample.

The cell counts of the CD45+ from the donors (n= 10) were determined before each DynaMAG and CEPir magnetic depletion and directly afterwards. The cells count can be different from one donor to another; the comparison has been done from the same sample and the same amount of starting materials.

Directly after the magnetic separations (depletion), the cells were labeled again with the anti-CD45 conjugated with PC5. The purities of the negative fractions from CD45 positive cells were analyzed using flow cytometry analysis (Figure 2).

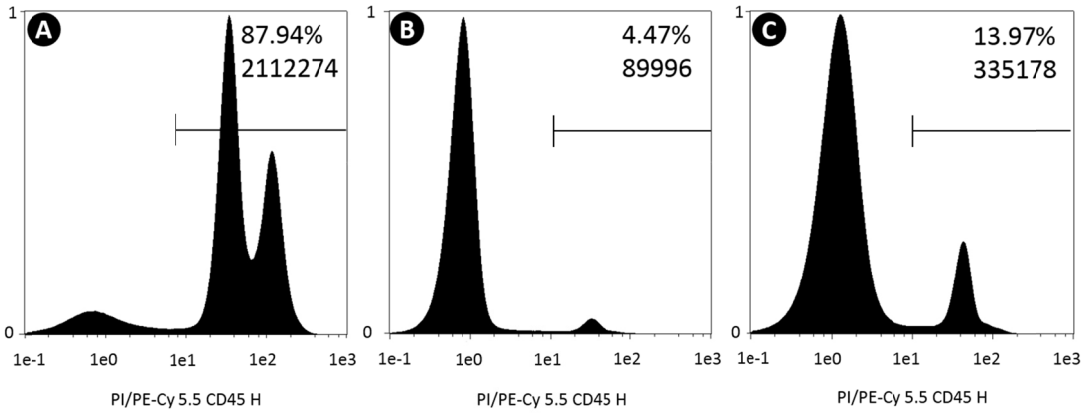

Figure 2. FACS analysis of CD45 cells in the negative fraction before and after the depletion using CEPir and DynaMag. CD45 expression in the control sample before depletion, two expression levels of CD45 in the control sample indicate different CD45 population (A). CD45 count (89996) and percent (4.47%) using the CEPir device (B). CD45 cell count (335178) and percent (13.97%) using DynaMag separator

For the CEPir device, the cells count of CD45+ in the negative fraction was significantly lower than the DynaMAG separator. For an example, in experiment number 7, the control sample showed total cells of 1,458,142; 61% of the total cells expressed CD45+ before the depletion, however, after using the DynaMAG depletion method, the sample showed 1.31% (31,502 CD45+ cells) in the negative fraction, while using CEPir technology, the sample showed only 0.35% of CD45+ and the cell number was lower as 8,362 cells (Fig 3). Based on the average of all the experiments, depletion with CEPir technology reduced the number of CD45+ cells to as low as 46.5%±21 than DynaMAG method.

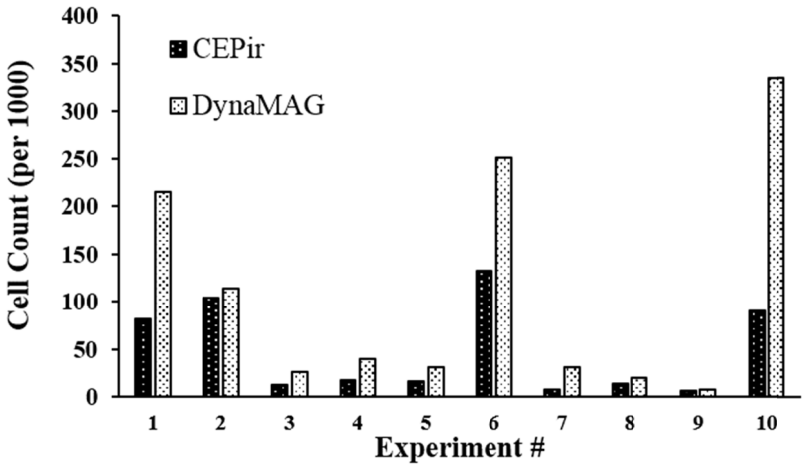

Figure 3. Different experiments of CD45 depletion (n=10). The number of CD45+ after CEPir and DynaMAG depletion

Due to the variation in the cells number before the depletion between each experiment, we evaluated and compared the efficiency of each method (Figure 4). The process efficiency is calculated according to the following formula:

Efficiency (%) =100 – (number of CD45 after depletion / number of CD45 before *100)

According to the Mann-Whitney Nonparametric Test, the depletion efficiency using CEPir technology was significantly higher (P=0.009) than DynaMAG method. CEPir technology was shown to effectively deplete CD45+ cells in peripheral blood samples.

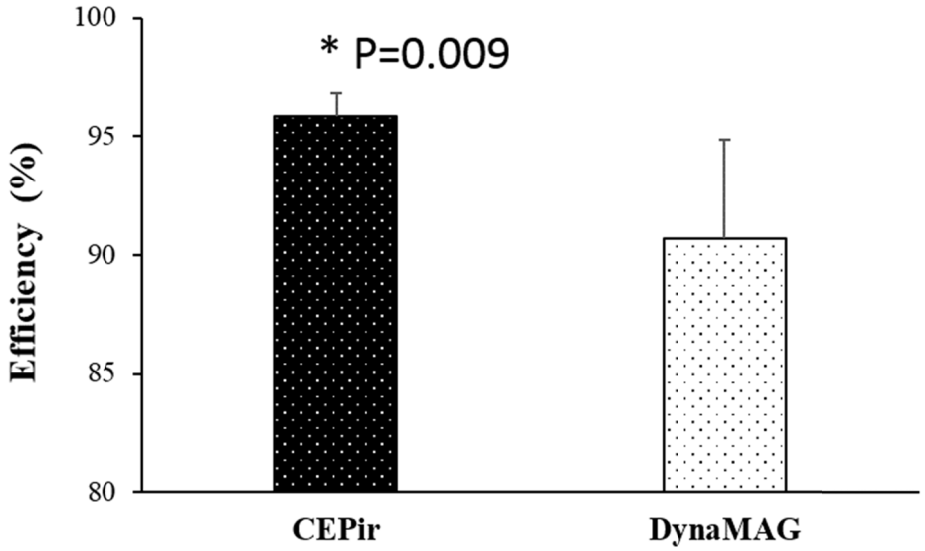

Figure 4. Comparison of the CD45 depletion efficiency of CEPir and DynaMAG technology (n=5)

4. Discussion

Today, more technologies and techniques exist for rare cells detection, but these technologies are lacking in specificity, sensitivity and reproducibility. More advent and sensitive technology is needed to improve the sensitivity threshold of rare cells detection. For example, CTCs are not detected in 50% of peripheral blood samples from metastatic cancer patients (Pantel, 2009).

Magnetic separation is relatively simple and fast compared to other methods; the cells isolated by magnetic separation process are usually pure, viable and unaltered. Different types of cells can be easily isolated directly from the peripheral blood but rare cells are mixed with the peripheral blood components and are thus rare, making their isolation and characterization a major challenge, in particular when capture of rare cells is based on antibodies labeling.

The major limit of detecting rare cells using magnetic beads is primarily influenced by the selection of specific markers on the cell population of interest, the expression level of those markers and the non-specific binding properties of the cell labeling reagents. Non-specific

labeling usually occurs when rare cells are flooded with various populations of other cells that increase the non-specific binding and reduce the cells' purity.

Therefore, we seek to reduce the effect of non-specific labeling by eliminating the non-target cells (CD45 cells) as a pre-enrichment step, this process is called negative selection platform. Previously, the negative selection and enumeration of circulating tumor cells was shown to be effective in other types of malignancies (head, neck and breast cancer) (Balasubramanian, 2012); (Balasubramanian, 2009); (Tong, 2007); (Yang, 2009).

Whenever the negative selection is more efficient, the detection of rare cells can be more effective. Therefore, we compared two different depletion methods (negative selection) using the same beads (anti CD45) with two different devices (DynaMag and CEPir).

CEPir showed lower number of CD45 cells in the negative fraction as compared to the DynaMag device, this indicates higher depletion of CD45 cells. In addition, the CEPir depletion efficiency was significantly higher than DynaMag (96% and 91% respectively).

This study elucidates the advantages of CEPir device by negative selection of CD45 cells as a pre-enrichment step to detect rare cells in peripheral blood sample. Additionally, negative selection offers opportunity for downstream application, since the target cells are untouched and unmodified by immunomagnetic reagents.

Advances in technology to isolate rare cells from peripheral blood or tissue will ultimately lead to better cellular, molecular characterization and innovation of new biomarkers. New technology and better performance will enable a wide range of disease evolution and clinical applications.

In conclusion, the negative selection (depletion) of leukocytes using CD45 marker is an effective strategy for the detection of rare cells using CEPir technology.

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