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P-selectin-coated microtube for the purification of CD45+ hematopoietic cells directly from human peripheral blood

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ABSTRACT

Purified samples of CD45+ hematopoietic cells are a prerequisite for chimerism analysis in transplantation therapies, and are useful in various research and clinical settings such as functional and molecular analysis or disease diagnosis. Recently, we have established a flow-based adhesion molecule-dependent process for the purification of these cells from human bone marrow. However, for practical purposes, it is desirable to apply this approach to process small volumes of human blood. CD45+ cell purities were >94% when PBMNCs and plasma depleted blood were perfused through P-selectin coated microtubes. However, P-selectin surface failed to capture CD45+ cells when fresh blood prior to washing was perfused. The process requires a pre-step of plasma removal which otherwise inhibits interactions of cell surface PSGL-1 with immobilized P-selectin due to the presence of soluble PSGL-1 in plasma. We conclude that P-selectin can be used in a compact flow device to isolate and purify CD45+ cells directly from human peripheral blood. The process is simple, rapid, cost effective and represents a physiologic approach to the capture and purification of CD45+ MNCs from peripheral blood.

Introduction

CD45 antigen is a type I transmembrane protein expressed on all hematopoietic cells except erythrocytes, plasma cells and platelets. Enrichment and purification of CD45-expressing hematopoietic cells (leukocytes) is important in transplantation therapies for hematological disorders as a prerequisite for chimerism analysis, especially in the setting of non-myeloablative and reduced intensity conditioning [1]. Further, these cells can be used in various research and clinical settings, such as for functional and molecular analysis, or disease diagnosis [2–4].

To date, immunomagnetic bead separation method (Miltenyi Biotech and R&D Systems) is the only available commercial approach which claims to purify CD45+ blood cells from human peripheral blood or bone marrow to a purity of >90%. However, binding of antibodies to cell surface antigens may prohibit cellular differentiation and proliferation [5]. Fluorescence-activated cell sorting (FACS) could be used as an alternative approach to obtain practically 100% pure CD45+ cells. However, this approach requires large amounts of initial samples, may pose contamination issues and cell sorters are prohibitively expensive for smaller scientific laboratories. By simply subjecting blood or marrow samples to Ficoll-paque density gradient separation followed by two washes with PBS, we are able to achieve up to 80% CD45+ cell purities and the purity may be enhanced to over 90% by incorporating an extra step of red blood cell lysis, however, such a lysis step could affect the viability of CD45+ cells [6].

Recently, by adopting a flow-based selectin dependent method, we were able to show significant capture and enrichment of CD34+ hematopoietic stem and progenitor cells from bone marrow and peripheral blood [7,8]. The technique is based on the verified hypothesis that mononuclear cells (MNCs) exhibit adhesive tethering followed by rolling on selectins expressed on endothelium, a key mechanism observed during inflammation [9–11]. Additionally, it has been shown that CD45+/CD34+ cells exhibit stronger rolling adhesion than CD45+/CD34− cells [7]. The technique works simply by perfusing samples through selectin-coated microtubes at wall shear stresses in the range of 1–3 dyn/cm^2. Recently, by implementing the same approach we have accomplished high purities (>90%) of CD45+ cells from human bone marrow samples [12]. However, for practical purposes peripheral blood as a starting sample is more convenient than aspirated bone marrow. Hence, in this manuscript by simply adopting a minor modification to the human peripheral blood sample, we show that it is possible to purify CD45+ cells from a small volume (100–250 µl) of blood by using a flow-based immobilized selectin approach. The method is rapid, simple, cost-effective and mimics a ubiquitous mechanism of cell trafficking in the body for recruitment of leukocytes during inflammation.

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Materials and methods

Cell capture microtube

Recombinant human P-selectin/Fc chimera (rhP/Fc) (R&D Systems) was coated onto the interior surface of micro-renathane tubing (MRE-025, ID 300 μm, length 50 cm, Braintree Scientific Inc) as described previously [7]. Briefly, the tubes were incubated with rhP/Fc (40 μg/ml in PBS) for 2 h at room temperature, followed by a gentle wash with PBS to remove unbound P-selectin. The tubes were then filled with calcium-enriched PBS (PBS+, pH 7.4) to activate immobilized P-selectin and stored overnight at 4 °C before perfusion. Control surfaces were prepared similarly except rhP/Fc was replaced with PBS.

Sample preparation

Peripheral blood samples (10 ml) were collected into heparin coated tubes from volunteer donors after informed consent in accordance with policies of the Research Subjects Review Board of the University of Rochester. Under sterile conditions, blood was diluted 1-fold with PBS free of calcium and magnesium. Peripheral blood MNCs (PBMNCs) were isolated by slowly pipetting 4 ml of diluted blood on 3 ml Ficoll-paque (GE Health Care) in 15-ml Falcon tubes. The tubes were centrifuged at 1800 rpm for 30 min at room temperature. The buffy layer containing PBMNCs was carefully collected in separate tubes, washed twice in PBS, and suspended in PBS+ at a concentration of 10^6 viable cells/ml. White cells were counted by diluting a fraction 3-fold with Turks solution (0.01% crystal violet and 3% acetic acid in distilled water) and counting on a hemocytometer after 3 min of incubation. Cell viability was assessed by trypan blue dye exclusion principle.

To separate plasma from the blood cells, blood was either incubated at room temperature for 30 min followed by decanting of the upper plasma layer or centrifuged briefly at 1000 rpm for 4 min followed by removal of the upper plasma layer. The pelleted blood cells were resuspended back to their original volume with PBS+. Plasma was further centrifuged at 2500 rpm for 10 min to remove residual debris and the clear supernatant was collected, aliquoted and frozen at −20 °C.

Cell capture, collection, and analysis

Microtubes were placed on the stage of an IX-71 inverted microscope (Olympus Inc) coupled to a CCD camera (Hitachi) and a TV monitor through a DVD recorder (Sony) for direct visualization of the captured cells on the inner tube surface. PBMNCs were perfused through the tube at a rate of 40 μl/min (wall shear stress 2.5 dyn/cm^2) for 10 min using a syringe pump system, followed by washing of the tubes with PBS+ for 20 min to remove nonadherent and loosely bound cells. Adherent cells were eluted by perfusing PBS-EDTA (10 μM) buffer through the tube at a rate of 8 μl/min (wall shear stress of 0.5 dyn/cm^2).

For quantitative flow cytometric analysis, a fraction of the sample was incubated with antibodies to CD45-Alexa 488 (Miltenyi Biotec) or isotype control-Alexa 488 for 20 min at 4 °C, washed twice with PBS and resuspended in 150 μl of PBS, and fluorescence data was acquired using a Guava EasyCyte flow cytometer (Millipore). FlowJo software was used to analyze the data.

Rolling studies

0.5 ml of PBMNCs (1×10^7 cells/ml) was either incubated with equal volumes of plasma or PBS-BSA (10%) for 10 min at room temperature. Viscosities of both samples were measured using an Ostwald's Viscometer. Samples were then perfused through P-selectin coated tubes on the IX-71 microscope stage at a wall shear stress of 2.5 dyn/cm^2 for 10 min followed by washing with PBS+ at the same shear. While washing, tubes were video recorded at random regions for approximately 1 min to calculate rolling velocities of the cells using a Matlab program as described previously [7].

Statistical analysis

Paired *t* test was used to analyze the results where appropriate, at the $\alpha=0.05$ level of significance.

Results

Capture and enrichment of CD45+ blood cells

As expected, perfusion of PBMNCs on the control surface resulted in negligible cell capture, whereas, perfusion of the same cells on the P-selectin coated surface exhibited a significant degree of capture. On average, surfaces coated with rhP/Fc (40 mg/l) captured approximately 100,000±17,100 MNCs, whereas control surfaces captured 1300±650 MNCs (Fig. 1a). These results indicated that tethering of PBMNCs on the surface is mediated by P-selectin and not due to nonspecific cell binding on the surface. Furthermore, previous studies perfusing bone marrow MNCs on IgG-coated control surfaces and anti-PSGL-1 antibody blocked P-selectin surfaces failed to capture cells on the surface, eliminating the possibility of nonspecific adhesion of cells to Fc receptor, and confirming the interaction of P-selectin-

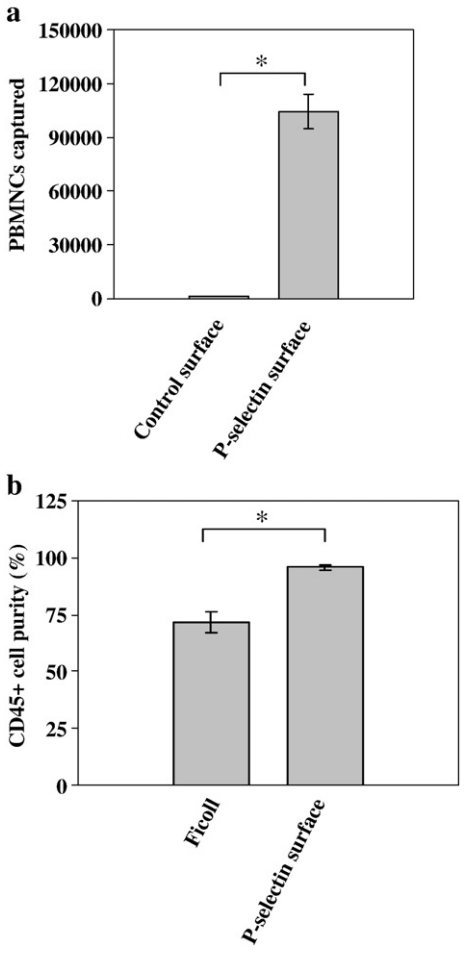

Fig. 1. Capture of PBMNCs on the surface of the P-selectin coated microtube. (a) The total number of cells captured on P-selectin surface and control surface at 2.5 dyn/cm^2 shear ($n=3$). (b) Purities (%) of CD45+ cells from Ficoll-extraction and P-selectin coated microtube ($n=3$). *$p<0.05$.