Journal of Chromatography A, 1373 (2014) 25–30

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Enrichment of circulating tumor cells using a centrifugal affinity plate system

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ARTICLE INFO

Article history:
Received 7 September 2014
Received in revised form 23 October 2014
Accepted 11 November 2014
Available online 18 November 2014

Keywords:
Circulating tumor cell (CTC)
Centrifugal force
Epithelial adhesion molecule (EpCAM)
High throughput

ABSTRACT

Circulating tumor cells (CTCs) are defined as cells that have detached from a primary tumor and are circulating in the bloodstream. Their isolation and quantification is of great value for cancer prognoses and drug testing. Here, the development of a centrifugal affinity plate (CAP) system is described, in which centrifugal force and antibody-based capture are exploited to enrich CTCs on one plate and hematological cells on the other. The CAP is rotated to exert centrifugal force on the cells in a blood sample, quickly transporting them to the anti-epithelial adhesion molecule (EpCAM)-coated and anti-CD45-coated surface of the CAP to shorten the reaction time and increase the adhesion force between the tumor and blood cells and each antibody. The effect of a rotating process on cell capture was investigated, and the capture efficiency was demonstrated using blood samples from healthy donors spiked with human non-small cell lung cancer (NCI-H1650) and breast cancer (MCF-7) cells. The CAP system was capable of rapid isolation and identification of CTCs without the requirement for pretreatment of blood samples. Finally, the CAP system was tested to evaluate the detection efficiency of CTCs in the blood samples of breast cancer patients. The number of captured CTCs in only 1 ml of blood varied from 6 to 10.

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1. Introduction

Circulating tumor cells (CTCs), which are cells that have escaped from a primary tumor and are circulating in the peripheral blood, invade distant organs and create new tumors when certain conditions arise in the microenvironment [1]. Thus, the presence of CTCs has been widely recognized as a representative marker for the evaluation of minimal residual disease after primary surgery [2]. The existence of CTCs is a rare event in the blood stream (1–100 CTCs/10^9 hematological cells); consequently, their isolation is extremely challenging. However, the precise detection and enumeration of these cells has caused a paradigm shift in cancer diagnosis and prognosis because CTCs, which are alternative sources of tumor cells that can be used for diagnosis or clinical study, can be accessed using a “liquid biopsy’ instead of an invasive biopsy [3].

To date, various technologies based on microfluidics have been developed to isolate CTCs among an enormous number of hematological cells, including size-based filtration [4], immuno-magnetic separation [5], dielectrophoresis (DEP) [6], and immuno-affinity micro-posts [7]. In the last decade, size-based platforms (based on the fact that epithelial-derived tumor cells are generally larger than hematological cells) and affinity-based platforms that utilize the protein markers expressed on the CTC surfaces, such as epithelial adhesion molecule (EpCAM), have been considered as promising tools for CTC research [8,9]. However, a recent study reported that CTCs and white blood cells (WBCs) overlap in size, so the smaller sizes of CTCs would be missed in the size-based separation of CTCs [10]. Furthermore, to reliably detect CTCs present in small number, a large sample volume of blood has to be examined with a high-throughput treatment [11,12]. Because of the high shear force, the CTCs captured by antigen–antibody reactions on a microfluidic channel surface tend to be detached from the channel surface at a high flow rate. The high-throughput issue raises problems not only due to this high shear force, but also due to the low Reynolds numbers associated with microfluidic techniques [13]. Since laminar flow is dominant in a microfluidic channel, the chance of a collision between CTCs and antibodies on the channel surface is low [14]. To increase the probability of such collisions, the flow must

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http://dx.doi.org/10.1016/j.chroma.2014.11.027
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be decreased to allow diffusion; however, this would require a very slow process for the large sample volumes of blood (i.e., more than 7.5 ml).

Here, we present a novel method that uses centrifugal force to reduce the processing time and enhance enrichment yield. The centrifugal affinity plate (CAP) system consists of an antibody-coated plate to capture cells and rotating equipment to generate centrifugal force. Antibody against the epithelial cell adhesion molecule (anti-EpCAM) was coated onto one plate (the CTC plate) to capture the CTCs, whereas antibody against CD45 (anti-CD45) was coated onto the other (the Hemato plate) to capture white blood cells. The CTC plate serves to enrich the CTCs from the blood, and it can be used directly for diagnosis and prognosis after fluorescence immunostaining. The Hemato plate is responsible for capturing the blood cells and consequently depletes the hematological cells when the plate is discarded. The CAP system can treat one to several milliliters of blood sample, suggesting that it can be useful for multiple samples or large sample volumes (Fig. 1).

2. Materials and methods

2.1. Design of centrifugal affinity plate (CAP) system and surface modification of CAP

Two slide glasses (25 mm × 75 mm) were used as the EpCAM and CD45 antibody-immobilized surface, respectively, and a chamber to accommodate the blood sample was fabricated from polycarbonate (PC; see Fig. 2). The sample was injected into the CAP by inserted the syringe needle in the inlet hole. The working volume of the chamber was varied from 1 to 5 ml. After sample injection, the CAP was located in the plate holder which is vertically fixed at the rotating stage, and the rotating stage generated the centrifugal forces to the CAP (0.3–26 G). The packaged CAP system has 20 cm in length, 30 cm in width, and 15 cm in depth.

A key advantage of the CAP is that all cells can simultaneously collide against the 600-mm^2 antibody-coated plate surface. Assuming that the average size of a single cell is a maximum of 20 μm, the number of cells attached to the capture surface can be estimated as 1.9×10^6. The glass surface of the CAP was treated with *S*-adenosyl-L-methionine (SAMe). First, the CAP was treated for 30 min with a solution of 1% aminopropyltriethoxysilane mixed with ethanol. The ethanol was then evaporated at 80 °C on a hot plate for 1 h. Protein was attached to the CAP processed with SAMe after the surface was treated with 3% glutaraldehyde in deionized (DI) water for 1 h. After being washed with phosphate-buffered saline (PBS) and DI water, the surface of the CAP was coated with anti-EpCAM or anti-CD45 antibody (10 μg/ml) in PBS for 30 min. After antibody treatment, the CAP was rinsed with PBS to wash out the non-binded antibodies. Finally, the PBS with 1% bovine serum albumin was injected into the CAP for 1 h to prevent any non-specific interactions.

2.2. Sample preparation and cell viability tests

The human non-small-cell lung cancer cells (NCI-H1650) and the human breast cancer cells (MCF-7) were incubated and grown to confluence in RPMI-1640 medium containing 1.5 mM L-glutamine supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2, with humidity, as suggested by the manufacturer. The cells were counted using a hemocytometer and serially diluted in PBS.

Blood samples were drawn from healthy donors without tumors or from metastatic breast cancer patients after obtaining informed consent at Severance Hospital of Yonsei University under an institutional review board-approved protocol. All specimens were collected in vacutainer tubes containing the anticoagulant ethylenediaminetetraacetic acid and were processed within 24 h. Whole blood specimens were stored at 4 °C on a rocking platform to prevent cell settling.

Because the shear forces in the process of centrifuge might damage the cell membrane, cell viability was determined with the LIVE/DEAD viability assay kit (AAT Bio-Quest Co.). This assay is based on the intracellular esterase activity of live cells and the plasma membrane integrity of dead cells. Briefly, captured CTCs on the CTC plate were incubated at room temperature for 30 min in a solution of 2 mM calcein fluorescein isothiocyanate (FITC) and 4 mM propidium iodide (PI) in PBS. At the end of the incubation period, the CAP was washed with 1 ml of PBS and visualized under a microscope. The live cells were stained with green fluorescence because calcein generated from Calcein-AM by esterase in a viable cell. Since the nuclei staining dye PI is not permeable to membrane of viable cell, only dead cells were stained with red fluorescence by passing through disordered areas of dead cell membrane.

2.3. Immunofluorescence staining to identify CTCs

Captured cells were fixed by flowing 1 ml of 4% paraformaldehyde (PFA) in PBS through the device for 20 min. The chip was subsequently washed with a solution of 1 ml of 0.2% Triton X-100 in PBS for 30 min to induce cellular permeability and allow for intracellular staining. To identify any bound WBCs, 1 ml of anti-CD45 (conjugated to FITC) stock solution was applied to the CAP for 2 h, followed by a PBS wash to remove excess antibody. In the same way, 1 ml of anti-cytokeratin [conjugated to phycoerythrin (PE)] stock solution was applied to the CAP to identify CTCs. Finally, to permit the identification of cellular nuclei, 1 ml of 4′,6-diamidino-2-phenylindole (DAPI) solution was applied to the chip for 5 min, followed by a PBS wash. The CAP was removed from the manifold, wiped dry near the fluid ports, and stored in the dark at 4 °C until imaging.

Biotinylated mouse anti-human anti-EpCAM was obtained from R&D Systems. The human non-small-cell lung cancer line NCI-H1650 and the Jurkat clone E6-1 blood cell line were purchased from the Korea Cell Line Bank, and RPMI-1640 growth medium was purchased from Invitrogen. Anti-cytokeratin (CAM 5.2, conjugated to PE), anti-CD45 (conjugated to FITC) and the fluorescent nucleic acid dye DAPI were purchased from BD Biosciences.

3. Results

3.1. System integration for the CAP, and surgical procedure for CTC isolation

Slide glasses coated with each antibody were tightly bonded with double-sided tape placed between the PC walls; these were named the centrifugal affinity plates (CAPs). A CAP was positioned in a plate holder (Fig. 1). Our system could rotate the CAPs from 0.3 to 26 G and was equipped with a plate holder (radius of rotating stage = 55 mm).

Centrifugal force is of great importance in our system to capture CTCs from human blood samples. Cells were forced to move to the antibody-coated surface of the plate due to centrifugal force. Fig. 1 shows three rotating steps to attach different cells to different CAPs (in this case, the Hemato plate for WBCs and the CTC plate for cancer cells). In step 1, centrifugal force was exerted on the surface of the slide glass coated with anti-CD45 antibody to capture WBCs. At that time, all of the cells moved to the surface of the Hemato plate. In step 2, the faces of the two CAPs (i.e., CAP1 and CAP2) had to be manually changed to the opposite direction for the cells to move toward the other plate surface via centrifugal force. In this step, almost all of the WBCs remained on the surface of the Hemato plate due to the affinity force of the anti-CD45 antibody–antigen reaction,

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