Cancer Letters 403 (2017) 216–223

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

Detection of circulating tumor cells from cryopreserved human sarcoma peripheral blood mononuclear cells

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ARTICLE INFO

Article history:
Received 13 March 2017
Received in revised form
2 May 2017
Accepted 11 May 2017

Keywords:
Sarcoma
Circulating tumor cells
Cell surface vimentin
Cryopreservation
Peripheral blood mononuclear cells

ABSTRACT

Circulating tumor cells (CTCs) enter the vasculature or lymphatic system after shedding from the primary tumor. CTCs may serve as “seed” cells for tumor metastasis. The utility of CTCs in clinical applications for sarcoma is not fully investigated, partly owing to the necessity for fresh blood samples and the lack of a CTC-specific antibody. To overcome these drawbacks, we developed a technique for sarcoma CTCs capture and detection using cryopreserved peripheral blood mononuclear cells (PBMCs) and our proprietary cell-surface vimentin (CSV) antibody 84-1, which is specific to tumor cells. This technique was validated by sarcoma cell spiking assay, matched CTCs comparison between fresh and cryopreserved PBMCs, and independent tumor markers in multiple types of sarcoma patient blood samples. The reproducibility was maximized when cryopreserved PBMCs were prepared from fresh blood samples within 2 h of the blood draw. In summary, as far as we are aware, ours is the first report to capture and detect CTCs from cryopreserved PBMCs. Further validation in other types of tumor may help boost the feasibility and utility of CTC-based diagnosis in a centralized laboratory.

Introduction

Sarcoma is a rare group of mesenchymal origin tumors, accounting for nearly 20% of pediatric malignancies and less than 2% of adult neoplasms [1,2]. Despite the low incidence of sarcoma, it represents a much larger proportion in adolescents and young adults with high mortality rate due to late diagnosis and relapse. A potential new approach for the early detection of relapse is to capture the circulating tumor cells (CTCs) from peripheral blood of sarcoma patients who are under remission. CTCs are “seed” cells for tumor metastasis that are shed into the circulatory or lymphatic system from the primary tumor [3,4]. These cells have attracted attention due to their potential role in early diagnosis and monitoring of therapeutic response to anti-cancer drugs [5–7]. At present, the CellSearch system is the only technique approved by the US Food and Drug Administration for the detection and enumeration of CTCs in metastatic breast, colorectal, and prostate cancers in the clinical setting [8–11]. CellSearch captures CTCs by utilizing the epithelial cell adhesion molecule (EpCAM) which, as its name suggests, is overexpressed only in epithelial cancer types [12,13]. However, this marker is not effective in capturing CTCs originating from mesenchymal tumors such as sarcoma, and might even miss some of CTCs undergoing epithelial–mesenchymal transition (EMT) [12,14]. Thus, a novel technique for accurately detecting CTCs from sarcoma patients' peripheral blood is quite necessary.

Abbreviations: CTCs, Circulating tumor cells; CSV, Cell-surface vimentin; PBMCs, Peripheral blood mononuclear cells; EpCAM, Epithelial cell adhesion molecule; EMT, Epithelial–mesenchymal transition.

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http://dx.doi.org/10.1016/j.canlet.2017.05.032
0304-3835/

Previously, we have reported that cell-surface vimentin (CSV) is a marker unique to different types of tumor cells [15–17]. By utilizing CSV as a specific target, we captured and enumerated mesenchymal-derived CTCs and EMT-like CTCs from fresh blood samples of patients bearing different types of cancer with high sensitivity and specificity [14,18]. However, to the best of our knowledge, the current CTCs capture techniques require fresh blood samples [19]. A reliable and reproducible cytometric technique for the enumeration of CTCs from cryopreserved samples is still lacking. Fresh samples have to be processed within 72 h as collection to maintain the reproducibility [20]. Transportation from multiple laboratories is not only expensive but also may affect the reproducibility of CTCs measurement. All the above barriers limit the application of current CTCs isolation techniques for large multiple-center trials. To boost CTCs assay utility, cryopreserved sample-based CTCs capture should be investigated.

In the current study, we investigated an assay for capturing CTCs from cryopreserved peripheral blood mononuclear cells (PBMCs) from patients with various types of sarcoma using the tumor specific CSV antibody 84-1. The new isolation step can be highly time limiting, which prevents large numbers of samples being processed on the same day. Such a technology will boost the feasibility and utility of CTC-based diagnosis and therapeutic treatment monitoring in large multiple-center trials.

Materials and methods

Patient eligibility and recruitment

Patients with metastatic cancer disease were consented in the Department of Laboratory Medicine and Sarcoma Center at The University of Texas MD Anderson Cancer Center. Blood was drawn either before or at least 7 days after intravenous therapy. Blood samples from healthy donors were obtained from Gulf Coast Blood Center in Houston, Texas. The healthy donors had no known disease or infection at the time of blood draw and no history of malignant disease. The study was approved by our institutional review board (Protocol: PA13-0353 and LAB06-0581). Informed and written consent was obtained from all the patients involved in this project.

Blood collection and cryopreserved PBMC preparation

Freshly drawn blood samples from patients were collected from patients in 10-mL BD Vacutainer tubes with K2 EDTA (BD Diagnostics Franklin Lakes, New Jersey) (Fig. 1A). A total of 500 mL of fresh blood from healthy donors was also collected. Blood was processed into 50 mL of Buffy Coat, which contained mononuclear cells and neutrophils, as well as smaller numbers of contaminating red blood cells, plasma, and platelets (Fig. 1B). All the blood samples were processed within 2 h of blood collection for best results. Obtained blood was pooled into 50-mL conical tubes and diluted with room-temperature phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Next, the diluted blood was layered carefully over 15 mL of Ficoll-Paque PLUS density gradient medium (Ficoll Paque Premium, GE Healthcare, Pittsburgh, PA) in a 50-mL SepMate™ tube (StemCell™ Technologies, Vancouver, Canada) while continuously pipetting the diluted blood down the side of the tube (Fig. 1C).

The samples were centrifuged at 1200× *g* for 10 min at room temperature with the brake on. After density gradient centrifugation, differential migration of cells during centrifugation resulted in the formation of layers containing different cell types. The bottom layer contained erythrocytes and granulocytes. PBMCs could be found together with other low-density slowly sedimenting particles (e.g., platelets) at the interface between the plasma and the Ficoll-Paque layer (Fig. 1C). The PBMCs were harvested by pouring the top layer, transferred to a new tube and then washed twice with PBS containing 2% FBS at room temperature. The cell pellet was resuspended with medium at room temperature. After cell counting, PBMCs were cryopreserved at 6×10^6 (6 million, nearly 6 mL of whole blood) cells per 1 mL and placed inside a Nalgene® Mr. Frosty® Cryo 1 °C Freezing Container (Thermo Fisher Scientific, Waltham, MA) container at −80 °C for 48 h, and the cells were later transferred to liquid nitrogen until further experiments were performed.

84-1+ cell selection

The selection and validation methods used to isolate and enrich 84-1+ cells were previously described [15]. Briefly, an antibody against human Fc receptor (Miltenyi Biotec, Auburn, CA) was added to the PBMCs cell suspension to reduce nonspecific binding. $CD45^+$ immune cells were then depleted using an EasySep human CD45 depletion kit (StemCell™ Technologies) according to the manufacturer's recommendation. Later, the CD45-cell fractions from the blood were labeled with the 84-1 anti-vimentin antibody, followed by the addition of mouse IgG-binding microbeads (Miltenyi Biotec) to the mixture after incubation. The labeled cells were then extracted using the magnetic column according to the manufacturer's protocol (Miltenyi Biotec). The 84-1+ and CD45-cells obtained were CSV + CTCs ready for further analysis (Fig. 1D).

Immunofluorescence imaging

For immunofluorescence, the cell pellet was mixed with MACS buffer (Miltenyi Biotec) and stained with 84-1 antibody (1:200) in tube at room temperature for 1 h. Cells were cytospun onto Polysine™ microscope adhesion slides (Thermo Fisher Scientific) by Cytofuge (Iris, Westwood, MA). Cells were fixed with 4% paraformaldehyde (Fisher Scientific) and blocked in blocking buffer (1% FBS in PBS) for 1 h. For the staining of other markers, such as CD45 (1:100; Abcam, Cambridge, MA), α-SMA (1:100; Abcam), CD117 (1:100; Cell Signaling Technology, Danvers, MA), and MDM2 (1:100; Santa Cruz Biotechnology Santa Cruz, CA), the cells were incubated with primary antibody overnight in a cold room followed by permeabilization in PBS (pH 7.4)/0.2% NP40 (Sigma Aldrich) for 20 min. Next, the slides were washed with PBS three times and stained with Alexa Fluor-555 secondary antibody (1:100; Invitrogen, Carlsbad, CA) for CD45, Alexa Fluor-647 (1:100; Invitrogen) for 84-1, and Sytox Green (1:200; Invitrogen) for nuclei staining for 1 h at room temperature. Then the slides were washed with PBS three times and mounted in Slow fade antifade (Invitrogen). All slides were visualized by Zeiss LSM 510 confocal microscope using LSM 5 3.2 image capture and analysis software (Carl Zeiss, Thornwood, NY).

Spiking assay

First, all cells used for the spiking assay were subjected to 84-1+ expression selection one day before the analysis. After being labeled with CFSE tracking dye (eBioscience, San Diego, CA), the cells were diluted to the required count numbers. For sensitivity analysis, 0, 5, or 25 LM7 cells (see below) labeled with CFSE were spiked into 6 million PBMCs (nearly isolated from 6 mL of whole blood). PBMCs with spiking cells were cryopreserved with an initial 1 mL of prepared ice-cold freezing media (90% FBS and 10% DMSO). Spiking assays were performed in triplicate to test the reproducibility of the cell recovery rate using this method.

LM7 cell line and culture conditions

Human osteosarcoma cell line LM7 was kindly provided by Dr. Eugenie S. Kleinerman (MD Anderson). LM7 cells were cultured in DMEM/F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS and 10 U/mL penicillin and streptomycin (Life Technologies, Carlsbad, CA) in an atmosphere of 5% CO_2 at 37 °C. Cells were subcultured every 2–3 days and harvested in the logarithmic phase of growth. Cell viability was assessed using the trypan blue dye (Life Technologies) assay. Live cells with viability greater than 98% were used for spiking assay experiments.

Statistical analysis

The average spiking cell recovery data were expressed as the mean ± standard deviation and representative results were from at least three independent experiments. The correlation between spiking cell count and recovered cell count, as well as reproducibility of CTC measurements, was assessed using liner regression and Bland-Altman analysis. For Bland-Altman analysis, the error of each CTC count was determined by the difference in the CTC count between two samples divided by the mean CTC count of both samples, and these errors were plotted on a graph. All statistical analyses were performed using GraphPad Prism software. P values less than 0.05 were considered significant.

Results

The modified technique enables capture of CTCs from cryopreserved PBMCs

We previously established a method in which enriched CSV-positive CTCs were stained with 84-1 antibody after fixation for immunofluorescence imaging [14]. However, this method was not a feasible approach for cryopreserved PBMC samples. CSV-positive CTCs, captured from cryopreserved samples, stained positive for 84-1 antibody both on the cell surface (CSV) and in the cytoplasm (intracellular vimentin; Fig. 2A). Our previous published results showed that CSV was specific to cancer cells and did not appear on normal immune cells occasionally co-captured with CTCs when using fresh blood samples. However, normal $CD45^+$ immune cells were also stained positively with CSV antibody when newly thawed PBMCs were used (Fig. 2B). It appeared that the thawed cells were too fragile to be membrane-permeabilized during spinning and fixation with paraformaldehyde. Thus, it was not feasible to