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Original Articles

A novel detection strategy for living circulating tumor cells using 5-aminolevulinic acid

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ABSTRACT

Most circulating tumor cell (CTC) detection methods have technical limitations, allowing the detection of only cells expressing epithelial antigens, and they cannot identify if the CTCs are alive or dead. Herein, we constructed a novel CTC detection system comprised of filter separation and 5-aminolevulinic acid (5-ALA)-based labeling, termed "Fs-ALA". Blood specimens (7.5 mL) were subjected to this method. Cells enriched on the filter were incubated with 5-ALA and Hoechst 33342 as positive markers for CTCs. Images of the whole filter surface were obtained using a fluorescence microscope. No 5-ALA positive cells were detected in healthy blood specimens. The Fs-ALA method was capable of detecting not only EpCAM-positive, but also EpCAM-negative tumor cells. In the Fs-ALA method, one or more CTCs were detected in samples from 13 of 18 (72.2%) colorectal cancer patients. The Fs-ALA method had a significantly higher CTC detection rate than CellSearch™ in colorectal cancer patients ($P < 0.05$), and only the former was capable of identifying live cells. This method is highly efficient for detecting CTC populations having undergone phenotypic changes, such as epithelial–mesenchymal transition.

Introduction

Circulating tumor cells (CTCs) have been associated with clinical outcome in various malignancies, such as breast [1], prostate [2], lung [3], gastric [4], and colorectal cancer [5,6]. General techniques for the enrichment of CTCs include immunomagnetic separation, filter separation, and density gradient separation. The more widely studied CTC detection methods are based on immunomagnetic enrichment with anti-EpCAM antibodies and subsequent immunological identification with anti-cytokeratin (CK) [7]. The CellSearch™ system utilizes a typical method and is the only system approved by the US Food and Drug Administration (FDA) for enumeration of CTCs in peripheral blood [8]. However, this method cannot detect EpCAM-negative CTCs, such as those having undergone an epithelial–mesenchymal transition (EMT), or detect if cells are alive due to requiring cell fixation for immunological detection.

As a new approach to identify living CTCs, we applied a photodynamic detection technique. This technique is based on the selective accumulation of photosensitizing molecules in living and malignant cells [9], which fluoresce following exposure to visible light. As a photosensitizing molecule, 5-aminolevulinic acid (5-ALA), a precursor to porphyrin in heme synthesis, has been used to stimulate endogenous protoporphyrin IX (PpIX) production in tumors [10]. 5-ALA is especially useful for the detection of living cells, because it is metabolized into PpIX, which emits red fluorescence. Due to the considerable differences in the activity of key enzymes in the heme pathway between tumors and normal tissue, PpIX accumulation induced by 5-ALA in tumor cells is higher than in normal cells [11]. Moreover, the induction of PpIX by 5-ALA was previously demonstrated to be diagnostically useful in various tumor diseases [12–14]. 5-ALA-based fluorescence has been detected in several cancers [15]. Recently, use of the 5-ALA-based approach resulted in the successful detection of not only primary lesions, but also metastatic sites [16,17]. Detection of metastatic cells in gastric cancer led us to develop a fluorescence microscopy approach to detect living CTCs using the 5-ALA-based approach.

In this study, we selected the filter separation method for CTC enrichment. The filter separation method is the most suitable for 5-ALA-based CTC labeling, because it is capable of separating living cells based on cellular physicality using no chemicals or antibodies [18]. Moreover, several studies have reported that the detection rate using the filter separation method was higher than using CellSearch [19–21]. Filter separation also has the potential to

Abbreviations: ALA, 5-aminolevulinic acid; Fs-ALA method, method composed of filter separation and 5-ALA-based labeling; PpIX, protoporphyrin IX; CK, cytokeratin.

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detect various populations of CTCs, such as mesenchymal-like cells that are subjected to EMT and down regulate the expression of epithelial markers, including EpCAM [22,23].

Herein, we report a novel strategy that integrates filter separation and 5-ALA-based CTC labeling, collectively called the Fs-ALA method. This method is capable of detecting enriched living CTCs in whole blood without any preprocessing, such as dilution, hemolysis, or partial purification, and is also applicable to various populations of CTCs regardless of epithelial marker expression.

Materials and methods

Cell culture and measurement of cell size distribution

The human colorectal tumor cell lines SW620, COLO 320DM, and SNUC1, and the human gastric tumor cell lines MKN7 and SNU1 were cultured in accordance with the specifications supplied by the American Type Culture Collection and Japanese Collection of Research Bioresources Cell Bank. All cells were grown in a culture dish to subconfluence prior to each experiment. Adherent cells were collected using 0.01% EDTA and 0.125% trypsin, and the collected cells were then washed with phosphate-buffered saline (PBS). Floating cells were collected and centrifuged, followed by washing with PBS. Cell density was measured using a hemocytometer and adjusted using appropriate buffer or medium. Cell size distribution was measured using a coulter counter (MoxiZ; ORFLO Technologies, Hailey, ID, USA).

Chemicals and antibodies

5-ALA (Cosmo Bio Co., Ltd., Tokyo, Japan) was freshly dissolved in PBS and used at a concentration of 1 mM for staining. Once taken up by cells, this substance is metabolized into protoporphyrin IX (PpIX), a fluorescent agent with a main absorption peak at 405 nm and a main fluorescence peak at 635 nm [24,25].

The following phycoerythrin (PE)-conjugated antibodies were used: anti-EpCAM (HEA-125; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and anti-pan-CK (C11; Abcam plc, Cambridge, UK). The following fluorescein isothiocyanate (FITC)-conjugated antibodies were used as negative markers: anit-CD45 (Miltenyi Biotec GmbH) and anti-CD31 (BD Biosciences, San Jose, CA, USA).

Evaluation of 5-ALA-based labeling performance in living human tumor cells

The collected cells were incubated with 1 mM 5-ALA in PBS at 37 °C for 75 minutes. After incubation, the 5-ALA solution was removed, and the cells were washed with PBS. Induction of PpIX fluorescence by 5-ALA was examined by fluorescence microscopy (TE2000; Nikon, Tokyo, Japan; excitation 395–410 nm and emission 610–640 nm). The intensity of PpIX fluorescence in the images was measured and analyzed for individual variability of 5-ALA metabolism with NIS-Elements microscope imaging software, NIS-Elements Analysis D ver. 4.00.06 (Nikon).

Hydrogen peroxide (H_2O_2) was used to induce cell death. MKN7 cells were treated with 100 mM H_2O_2 in culture medium or were left untreated. After incubation at 37 °C and 5% CO_2 for 60 minutes, the cells were washed with PBS to remove H_2O_2. The cells were then stained with 5-ALA as described above. For determination of cell viability, the cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). Both H_2O_2-treated and untreated cells were examined with a fluorescence microscope and analyzed with microscope imaging software under the same conditions and settings.

Blood specimen collection

All patients were enrolled in a protocol (Registry number: 2012-1012) approved by the Institutional Review Board at the Cancer Institute Hospital of the Japanese Foundation for Cancer Research (JFCR). Patients who had a histologically confirmed diagnosis of unresectable and recurrence, adenocarcinoma of colorectal cancer and gastric cancer and squamous cell carcinoma of esophageal cancer were eligible for enrollment. Additional eligibility criteria included general condition (Eastern Cooperative Oncology Group performance status 0–2) and written informed patient consent. Written informed consent was obtained from all patients. Additionally, healthy volunteers who were men and women of 30–48 years of age, and had no history of cancer provided informed consent prior to participation in this study. For assay using the Fs-ALA method, whole blood samples were drawn from patients with colorectal, gastric, and esophageal cancer or healthy volunteers using vacutainer tubes containing EDTA·2K and were analyzed for experiments within 6 hours. For assay using the CellSearch system (Veridex LLC, Raritan, NJ, USA), 10 mL samples of blood were drawn into a CellSave Preservative Tube (Veridex LLC).

CTC enrichment and labeling system

The CTC enrichment system consisted of a unit equipped with size-selective microfilter (filter unit), three types of reservoirs (to feed blood specimen, to wash flow channel and filter, and to inject reagent), and a waste tank. The driving force to send the solution was generated by a constant pressure source, which was controlled by a head differential between the inlet and outlet port. Detailed information on the system and the microfilter is described in Appendix S1.

Evaluation of recovery rate by CTC enrichment system

The recovery rate of tumor cells in blood using our CTC enrichment system was evaluated using whole blood spiked with fluorescently-labeled human tumor cell lines. Detailed procedures are described in Appendix S1.

CTC enumeration by 5-ALA-based labeling method integrated with the CTC enrichment system

After blood specimens were filtered using our CTC enrichment system, the cells trapped on the size-selective microfilter were labeled using 5-ALA, Hoechst 33342, and negative markers (anti-CD45 and anti-CD31 antibodies labeled with FITC). The fluorescence images of labeled cells were analyzed with NIS-Elements Analysis D ver. 4.00.06. The 5-ALA-induced PpIX (+)/nuclei (+)/FITC (−) cells with cell-like morphology were identified as CTCs and were enumerated. Detailed procedures are described in Appendix S1.

Confirmation and comparison of detection performance to CellSearch system

Approximately 200 cells from the COLO 320DM and SW620 cell lines were spiked into 7.5 mL of whole blood collected using vacutainer tubes containing the anticoagulant EDTA for the Fs-ALA method, and using CellSave Preservative Tubes for the CellSearch system. Enumeration using Fs-ALA was conducted as described above. The CellSearch procedure has been previously described in detail [4]. Detection rates was analyzed separately before any results were known and comparisons were made between Fs-ALA and CellSearch. Additionally, the blood specimens collected from healthy volunteers were tested the same way as negative controls.

Enumeration of CTCs from clinical blood specimen

CTCs in clinical blood specimens (7.5 mL) collected from a total of 35 patients, consisting of 18 colorectal, 13 gastric, and 4 esophageal cancer patients, were assayed using the Fs-ALA method. To evaluate the sensitivity of Fs-ALA, the 35 specimens were assayed using both the Fs-ALA and CellSearch systems, and the detection rates were compared in a blinded experiment (CTCs were counted separately in accordance with the criteria before any results from CellSearch were known).

Statistical analysis

Fisher's exact test and Mann–Whitney U test were performed to assess statistically significant differences; a P-value < 0.05 was considered statistically significant.

Results

Staining performance of 5-ALA in human tumor cells

The efficiency of tumor cell labeling using 5-ALA was tested with five tumor cell lines in which EpCAM and cytokeratin expression levels differ. Fig. 1a shows that every type of tumor cell line showed clear fluorescence following incubation with 5-ALA. The EpCAM-negative tumor cell lines were COLO 320DM and SNU1. In EpCAM-positive tumor cell lines, the expression of SNUC1 and the others (SW620 and MKN7) were medium and high, respectively (Fig. 1b). Regardless of EpCAM expression level, all tumor cells were labeled with over 90% detection efficiency (Fig. 1a and c). These results suggest that 5-ALA derived PpIX signal was fully detected with 635 nm wavelength. In contrast, most of the leukocytes isolated from human peripheral blood were not labeled using 5-ALA (Fig. 1b). To determine whether 5-ALA-induced PpIX was detected only in living cells, the MKN7 cells were double-stained using 5-ALA and DAPI in a live or dead state (Fig. 1d). The dead cells that were treated with H_2O_2 and stained with DAPI provided no red fluorescence, whereas untreated (living) cells showed no blue fluorescence derived from DAPI and were stained using 5-ALA.