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The use of fluorescent indoline dyes for side population analysis

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ABSTRACT

Dye efflux assay evaluated by flow cytometry is useful for stem cell studies. The side population (SP) cells, characterized by the capacity to efflux Hoechst 33342 dye, have been shown to be enriched for hematopoietic stem cells (HSCs) in bone marrow. In addition, SP cells are isolated from various tissues and cell lines, and are also potential candidates for cancer stem cells. However, ultra violet (UV) light, which is not common for every flow cytometer, is required to excite Hoechst 33342. Here we showed that a fluorescent indoline dye ZMB793 can be excited by 488-nm laser, equipped in almost all the modern flow cytometers, and ZMB793-excluding cells showed SP phenotype. HSCs were exclusively enriched in the ZMB793-excluding cells, while ZMB793 was localized in cytosol of bone marrow lineage cells. The efflux of ZMB793 dye was mediated by ATP binding cassette (ABC) transporter Abcg2. Moreover, staining properties were affected by the side-chain structure of the dyes. These data indicate that the fluorescent dye ZMB793 could be used for the SP cell analysis.

1. Introduction

The side population (SP) cells, a small population of efficient Hoechst 33342 dye-excluding cells from diverse adult tissues, are highly enriched for stem cell activity [1]. Hoechst 33342 efflux strategies are originally described for the detection of hematopoietic stem cells (HSCs) in murine bone marrow [2,3]. The bone marrow SP cell population is present in the bone marrow of all species examined [4]. Murine skeletal muscle, brain, testis, mammary gland, lung, heart and embryonic stem (ES) cells also contains SP cells [5–14]. In addition, SP cells were identified in human cancer cells [15–17]. Because of the conserved SP phenotype in a wide range of different types of stem cells, dye efflux assay evaluated by flow cytometry is useful for stem cell studies.

The efflux of Hoechst 33342 was attributed to the member of ATP binding cassette (ABC) transporter of ATP-dependent cell surface proteins. Among various ABC transporter proteins, sub-family G, member 2 (Abcg2; also known as breast cancer resistance protein [Bcrp]) and sub-family B, member 1A/1B (Abcb1a/1b; also known as multidrug resistance [Mdr1a/1b] or P-glycoprotein [P-gp]) are important determinants of the SP phenotype, though the relative contributions of Abcg2 and Abcb1a/1b to the SP phenotype differ in each tissues [18]. In bone marrow, the major determinant of the SP phenotype is shown to be *Abcg2* through loss- and gain-of-function analyses [12,19]. *Abcg2* was originally cloned from a breast cancer cell line selected for its unique drug resistance in the presence of a P-gp inhibitor, verapmil [20]. Abcg2 was shown to exclude the chemotherapeutic agent [19], indicating that this transporter may function as a general protectant against endogenous and exogenous substances in stem cells [21].

Ultra violet (UV) light is required for optimal excitation of Hoechst 33342. Recently, another fluorescent Abcg2 substrate DyeCycle Violet (DCV), which can be excited by 405-nm violet light, was reported to identify the almost same population as did Hoechst 33342 [22–24]. However, even though 405-nm diode laser has become increasingly popular, these UV and violet lights are not as common as 488-nm argon laser in flow cytometer [25]. On the other hand, almost all the modern flow cytometers are equipped with the 488-nm laser, used for excitation of fluorescein isothiocyanate (FITC), green fluorescent protein (GFP), phycoerythrin (PE) and its tandem conjugates, and propidium iodide (PI).

According to the recent report, fluorescent indoline derivative ZMJ018 is recognized as a substrate for the efflux transporters in blood–brain barrier (BBB) and blood–retinal barrier (BRB) and the interaction with efflux transporter may be affected by its substructure [26]. In this study, we identified a fluorescent indoline dye ZMB793, which is a structurally similar compound of ZMJ018, adequately excited at 488 nm and excluded from murine primitive

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hematopoietic cells including HSCs. We showed the relationship between SP cells and ZMB793-excluding cells based on double-staining of the cells by ZMB793 and Hoechst 33342. Enrichment of HSCs by ZMB793 efflux assay was evaluated in terms of the surface membrane antigen phenotype and the colony formation activities. We also investigated the intracellular localization of ZMB793 by confocal microscopy. The active efflux of ZMB793 from bone marrow cells was investigated by examining the effect of ABC transporter inhibitors. Furthermore, the change in the staining property dependent on the side-chain structure of indoline dyes was tested by using several analogs of ZMB793.

2. Materials and methods

2.1. Materials

Fetal bovine serums (FBS) were purchased from Hyclone (Logan, UT, USA) and Stem cell technologies (Vancouver, BC, Canada). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from nacalaitesque (Kyoto, Japan). The monoclonal antibodies 2B8 (anti-c-Kit), D7 (anti-Sca-1), RAM34 (anti-CD34) were purchased from BD Bioscience (Rockville, MD, USA), HM48-1 (anti-CD48), TC15-12F12.2 (anti-CD150), 145-2C11 (anti-CD3e), 53-6.7 (CD8a), RA3-6B2 (anti-B220), 1A8 (anti-Ly6G), M1/70 (anti CD11b) were from BioLegend (San Diego, CA, USA). TER119 (anti-erythrocyte-specific antigen) and human erythropoietin were purchased from eBioscience (San Diego, CA, USA). TO-PRO 3 was purchased from Invitrogen (Carlsbad, CA, USA). 2.4G2 hybridoma was purchased from American Type Culture Collection (Rockville, MD, USA). Fumitremorgan C (FTC), digoxin, and probenecid were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). S-clone SF-O3 medium was purchased from Sanko Junyaku Co. (Tokyo, Japan). Bovine serum albumin and β-mercaptoethanol were from Sigma–Aldrich Co. (St. Louis, MO, USA). Mouse stem cell factor (SCF), Human thrombopoietin, mouse interleukin (IL)-3, and human erythropoietin were from R&D systems Inc. (Minneapolis, MN, USA).

2.2. Mice

C57BL/6NCrSlc female mice were purchased at 6–8 weeks old from Shimizu Laboratory Supplies Co. (Kyoto, Japan) and were used between 8 and 12 weeks old. All animal experimentation was conducted in accordance with the guidance of the Institute for Frontier Medical Sciences, Kyoto University.

2.3. Fluorescent indoline derivatives

All the fluorescent indoline dyes examined were obtained from Canon Inc. (Tokyo, Japan). Stock solutions of the fluorescent indoline dyes were prepared by dissolution in dimethyl sulfoxide (DMSO) at 1 mM. The excitation wavelength and the fluorescence wavelength of the fluorescent dyes were obtained by measuring DMSO solution containing 5 μM fluorescent dyes by FL4500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan).

2.4. Preparation of bone marrow cells

Fresh bone marrow cells were harvested from femurs and tibias, and were suspended in Hanks-balanced salt solution (HBSS) supplemented with 2% FBS (Hyclone), 10 mM HEPES, and penicillin/streptomycin (HBSS+). The cell suspensions were filtered through a cell strainer to remove debris. The filtrate was suspended in ice-cold HBSS+ and then pelleted by centrifugation for 5 min at 4 °C. The bone marrow cells were resuspended at 1×10^6 cells/ml in HBSS+.

2.5. Dye labeling

Mouse bone marrow cells were resuspended at 10^6 cells/ml in HBSS+ and labeled with 0.01–1 μM ZMB793 or its analogs in HBSS+ at 37 °C for 5–60 min. For the inhibitor experiments, FTC, digoxin, or probenecid was added to cells at indicated concentration. 1 μM ZMB793 was then added and incubated at 37 °C for 30 min. In other experiments, cells were incubated in the presence of Hoechst 33342 at 37 °C for 30 min. Then ZMB793 were added to give final concentration of 1 μM and incubated at 37 °C for additional 30 min. After staining, the cells were washed and resuspended in ice-cold HBSS+.

2.6. Flow cytometry

For immunostaining, the single-cell suspensions were blocked by the culture supernatant of 2.4G2 (anti-CD16/CD32) hybridoma, and stained with monoclonal antibodies in PBS containing 2% FBS (Hyclone) and 0.1% sodium aside. TO-PRO 3 was used to distinguish dead cells from viable cells. The immuno-stained cells were analyzed on a FACSCanto II flow cytometer equipped with 488-nm, 633-nm, and 405-nm laser or sorted on a FACSAria II cell sorter equipped with 488-nm, 532-nm, 640-nm, 405-nm, and 355-nm lasers (BD Biosciences). Analysis was performed by BD FACSDiva software (BD Bioscience) and FLOWJO software (Tree Star, San Carlos, CA).

2.7. Confocal microscopy

The bone marrow cells labeled with ZMB793 and TO-PRO 3 were sorted directly onto four-well chambered cover glasses (Lab-Tek™ Chambered Cover glass, Nalge-Nunc International, Rochester, NY, USA). Confocal microscopy was performed on an LSM 510 META using a 10×/0.3 NA or 63×/1.4 NA oil immersion objective lens (Carl Zeiss, Oberkochen, Germany). The cells were excited by 488-nm laser, and LP650 filter was used to detect fluorescence emission of ZMB793. All acquired images were processed with the LSM Image Browser (Carl Zeiss).

2.8. Single-cell colony assay

The single-cell colony assay was performed as described previously [27]. Briefly, the cells were sorted clonally into 96-well plates containing 100 μl of S-clone SF-O3 medium supplemented with 10% FBS (Stem cell technologies), 1% bovine serum albumin, 10 ng/ml mouse SCF, 10 ng/ml human thrombopoietin, 10 ng/ml mouse IL-3, 1 U/ml human erythropoietin, and 5×10^{-5} M β-mercaptoethanol. After 14 days, colonies containing more than 1000 cells were identified microscopically ($n = 3$ for each cell type).

2.9. Statistic analysis

All the results were expressed as the mean ± standard deviation (SD). Significant analysis between the experiment groups was done based on the one-way ANOVA, and the difference was considered to be significant at $p < 0.05$.

3. Results

3.1. Characteristics of fluorescent indoline dyes

Fig. 1A shows the chemical structures of ZMB793 (molecular weight, 621.79). Fig. 1B shows the excitation and emission spectra

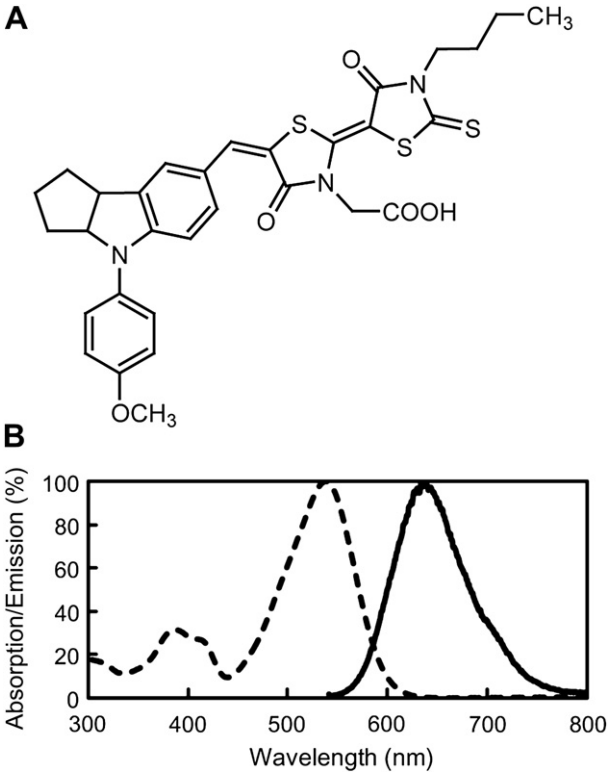

Fig. 1. Structure and excitation/emission spectra of ZMB793 dye. (A) Chemical structure of ZMB793. (B) Excitation (dashed line) and emission (solid line) spectra of ZMB793 measured at 5 μM in DMSO.