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RESEARCH PAPER

Single-cell sphingosine kinase activity measurements in primary leukemia

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Abstract Sphingosine kinase (SK) is a promising therapeutic target in a number of cancers, including leukemia. Traditionally, SK has been measured in bulk cell lysates, but this technique obscures the cellular heterogeneity present in this pathway. For this reason, SK activity was measured in single cells loaded with a fluorescent sphingosine reporter. An automated capillary electrophoresis (CE) system enabled rapid separation and quantification of the phosphorylated and nonphosphorylated sphingosine reporter in single cells. SK activity was measured in tissue-cultured cells derived from chronic myelogenous leukemia (K562), primary peripheral blood mononuclear cells (PBMCs) from three patients with different forms of leukemia, and enriched leukemic blasts from a patient with acute myeloid leukemia (AML). Significant intercellular heterogeneity existed in terms of the degree of reporter phosphorylation (as much as an order of magnitude difference), the amount of reporter uptake, and the metabolites formed. In K562 cells, the average amount of reporter converted to the phosphorylated form was 39±26 % per cell. Of the primary PBMCs analyzed, the average amount of phosphorylated reporter was 16±25 %, 11±26 %, and 13±23 % in a chronic myelogenous leukemia (CML) patient, an AML patient, and a B-cell acute lymphocytic leukemia (B-ALL) patient, respectively. These experiments demonstrated the challenge of studying samples comprised of multiple cell types, with tumor blasts present at 5 to 87 % of the cell population. When the leukemic blasts from a fourth patient with AML were enriched to 99 % of the cell population, 19±36 % of the loaded sphingosine was phosphorylated. Thus, the diversity in SK activity remained even in a nearly pure tumor sample. These enriched AML blasts loaded significantly less reporter (0.12±0.2 amol) relative to that loaded into the PBMCs in the other samples (≥1 amol). The variability in SK signaling may have important implications for SK inhibitors as therapeutics for leukemia and demonstrates the value of single-cell analysis in characterizing the nature of oncogenic signaling in cancer.

Keywords Single-cell analysis · Sphingosine kinase · Capillary electrophoresis · Leukemia · Primary cells

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Introduction

Sphingosine kinase (SK) is an important regulator of cell fate that converts sphingosine, a lipid signaling molecule, which induces apoptosis, to sphingosine-1-phosphate (S1P), which promotes cell survival, proliferation, and migration [1–3]. Because of its central role in regulating cell survival, S1P overexpression has been implicated in a number of cancers, including leukemia, prostate, breast, colon, pancreatic, and lung cancers [4, 5]. Leukemia is one of the most prevalent cancers, and is the deadliest cancer for people below the age of

20 in the USA [6]. Targeting the SK pathway has been effective at inducing apoptosis in multiple types of leukemic cells [7]. Inhibition of SK in cultured and primary leukemic cells also increases their sensitivity to chemotherapy, even in cells that have become multi-drug resistant [8, 9]. Notably, SK inhibitors induce apoptosis in primary acute myeloid leukemia (AML) cells at a much higher rate than in healthy cells [8]. SK is also overexpressed in chemotherapeutic-resistant chronic myelogenous leukemia (CML) cell lines [10] and is thought to regulate drug resistance by increasing the stability of Bcr-Abl, the oncogenic fusion protein that is the proximate cause of CML [11]. Drugs that target SK activity in cancer are currently in multiple clinical trials and are especially promising for treating drug-resistant leukemia [12].

One challenge facing the development of novel cancer therapeutics is that cells from the same tumor can have extraordinary phenotypic and functional heterogeneity [13, 14]. Intratumor heterogeneity has been demonstrated to have profound implications for disease evolution, patient prognosis, and the development of drug resistance [15]. Despite the important implications of tumor heterogeneity in treating leukemia [16–18], very little is known about the variability of SK activity at the single-cell level in primary leukemia. Conducting single-cell lipid kinase measurements in primary cells is difficult due to the challenges in generating antibodies specific for endogenous lipids, limited availability of patient samples, and a lack of robust technologies with single-cell limits of detection. Microscopy and flow cytometry are powerful methods that have been commonly used for single-cell analysis. In particular, phospho-specific flow cytometry, which utilizes antibodies against phosphorylated proteins, has enabled characterization of the activity of many kinases in primary samples [19, 20]. However, without commercially available antibodies specific to sphingosine and S1P, SK activity cannot be directly measured using these techniques. In addition, flow cytometry requires large numbers (10^4–10^6) of cells, making the method unsuitable for patient specimens with small numbers of cells [21]. Microscopy has been utilized to measure single-cell enzyme activity with fluorescent protein probes, but these probes must be genetically encoded, which limits their application in primary cells [22]. Separation techniques capable of physically distinguishing sphingosine and S1P, such as high-performance liquid chromatography with mass spectrometry detection, are commonly used to measure sphingolipids in ensemble samples of hundreds of thousands of cells, but the limits of detection are far too high to detect these analytes in single cells [23].

Capillary electrophoresis (CE) with laser-induced fluorescence detection is a promising technology for kinase activity measurements in single, primary cells. Typical CE limits of detection for fluorescent species are in the sub-attomole level, sufficiently low to quantify molecules from single cells [24, 25]. Single-cell CE has been utilized to measure lipid metabolism and enzyme activity in a number of cell types [26–28]. However, the majority of single-cell CE research has used immortalized cell lines for enzyme activity measurements. Although these cell lines are a convenient and often useful model system, there is increasing evidence that they may not resemble primary cells in terms of function, behavior, and physiological signaling [29]. Indeed, among the few studies that have performed single-cell CE analysis of enzymatic activity in primary cells, including rat brain neurons [30], human airway epithelial cells [31], and human pancreatic tumor cells [32], at least two found differences in activity between primary cells and cultured cells [31, 32]. There is a clear need for studies applying CE measurements to primary cells to increase the availability of this technology for clinically relevant measurements on cells.

This paper will describe the use of automated single-cell CE to characterize SK activity in tissue-cultured cells of leukemic origin, peripheral blood mononuclear cells (PBMCs) from leukemia patients, and purified leukemic blasts. Assay performance was optimized in the leukemia-derived K562 cell line. The metabolism of the fluorescent sphingosine was then quantified in these cells. Ensemble measurements of frozen and fresh PBMCs from patients with leukemia were compared to determine whether freezing cells altered SK activity in the primary cells. Phosphorylation of the SF reporter was then measured in single PBMCs, which were composed of a mixed population of leukemic blasts and normal leukocytes. Reporter uptake, phosphorylation, and the number of fluorescent metabolites in each cell were measured in the PBMCs from CML, AML, and B-ALL patients. SK activity was also measured in isolated leukemic blasts from a fourth patient with AML. In total, over 100 patient cells were analyzed and SK heterogeneity within each patient was characterized.

Experimental

Reagents and materials

Sphingosine 5/6-fluorescein (SF) and sphingosine-1-phosphate 5/6-fluorescein (S1PF) were purchased from Echelon Biosciences Inc. (Salt Lake City, UT). Sodium chloride, potassium chloride, magnesium chloride, calcium chloride, piperazine-1-ethanesulfonic acid (HEPES), and glucose were acquired from Sigma-Aldrich Inc. (St. Louis, MO). Monosodium phosphate, 1-propanol, methanol, isopropanol, and pre-cleaned glass slides (50 mm×45 mm×1.5 mm) were procured from Fisher Scientific (Pittsburgh, PA). Ethanol was acquired from Decon Labs (King of Prussia, PA). Roswell Park Memorial Institute Media (RPMI) was purchased from Cellgro (Manassas, VA) and Adoptive Immunotherapy Media (AIM-V®) was acquired from Gibco (Grand Island, NY).

Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA), human AB serum (HS) from Gemini BioProducts, and penicillin/streptomycin from Gibco (Grand Island, NY). EPON resin 1002F (phenol, 4,4′-(1-methylethylidene) bis-, polymer with 2,2′-[(1-methylethylidene) bis (4,1-phenyleneoxymethylene)]bis-[oxirane]) was procured from Miller-Stephenson (Sylmar, CA, USA). SU-8 developer (1-methoxy-2-propyl acetate) was acquired from MicroChem (Newton, MA, USA). Poly(dimethylsiloxane) (PDMS) (Slygard 184 Silicone Elastomer) was purchased from Dow Corning (Midland, MI).

Fabrication of three-channel device for automated single-cell CE analysis

The three-channel system for physiologic buffer, air, and electrophoretic buffer was fabricated from PDMS (Figure S1 Electronic Supplementary Material) [33]. The channel for loading electrophoretic buffer was 3 cm in length, 0.5 cm in width, and 1.5 mm in depth. The physiologic buffer channel was 3.5×0.3 cm, with circular reservoirs that were 1 cm in diameter at both ends of the channel. The electrophoretic and physiologic buffer channels were connected by a perpendicular 5×1 mm channel that was filled with air. The PDMS channels were bonded to a 1002F-coated coverslip containing an array of 10×10 microwell cell traps [34]. The cell trap array was placed at the center of the physiologic buffer channel. The cell traps used to capture K562 and primary cells were 30 and 15 μm in diameter, respectively. All cell traps were 20 μm in depth and separated from adjacent traps by 100 μm. The arrays of cell traps were fabricated using standard photolithographic procedures. Coverslips were washed with water, ethanol, and then dried using nitrogen gas. Coverslips were then plasma-cleaned for 45 min (PDC-001, Harrick), spin-coated with 1002F-10 for 30 s at 1,100 rpm, and soft-baked for 30 min at 95 °C. The 1002F-coated coverslip was then covered with an iron oxide photomask and illuminated with a total dose of 600 mJ of light using a UV exposure system (Oriel, Newport Stratford, Inc., Stratford, CT). The photoresist-coated coverslip was subsequently baked at 95 °C for 8 min, developed for 2 min in SU-8 developer, rinsed with isopropanol, and then hard-baked for 30 min at 120 °C.

Isolation of primary peripheral blood mononuclear cells from whole blood

De-identified patient peripheral blood samples were obtained from the University of North Carolina Hematolymphoid Malignancies Tissue Procurement Facility under an institutional IRB-approved protocol in accordance with the Declaration of Helsinki. The Tissue Procurement Facility additionally provided information about the percentage of leukemic cells for each patient. Peripheral blood samples were stored at room temperature and processed using Ficoll-Paque PLUS density centrifugation within 24 h after extraction. Briefly, 5 mL of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Pittsburgh, PA) was added to a sterile 15-mL centrifuge tube (Denville Scientific Inc., Metuchen, NJ). Eight milliliters of whole blood diluted at least 1:1 in phosphate-buffered saline (PBS) was gently layered on the Ficoll-Paque PLUS, so as not to disturb the interface between the liquids. The tubes were centrifuged for 20 min at 900×g in a swinging bucket centrifuge with the brake off. PBMCs were collected from the interface of the two layers and immediately washed twice with PBS.

Cell culture

K562 cells, which were derived from a CML patient in blast crisis, were grown in RPMI supplemented with 10 % FBS, 50 mg/mL streptomycin, and 50 units/mL penicillin. Frozen K562 cells were thawed and passed for 1 week before being utilized in single-cell experiments. K562 cells were not used in assays past their 15th passage. Primary cells were maintained in AIM-V® containing 10 % heat-inactivated HS and 1 % penicillin/streptomycin. Fresh primary cells were analyzed within 6 h of isolation from whole blood. Between experiments, primary and cultured K562 cells were stored at 37 °C in a humidified incubator with 5 % carbon dioxide.

Cell viability measurements

Viability was determined using a trypan blue exclusion assay. Cells were pelleted, resuspended in PBS, and stained with a final concentration of 0.35 % trypan blue. Viable cells were counted using a hemocytometer 2–3 min after the addition of the trypan blue stain. At least 100 cells were counted for each viability determination. The number of cells per unit volume of buffer was determined by counting viable cells using a hemocytometer.

Enrichment of $CD34^+$ AML blasts from PBMCs

Selection of $CD34^+$ cells from Ficoll-Paque PLUS isolated PBMCs was performed using the CD34 MicroBead Kit UltraPure (Miltenyi Biotec, Inc.) following the manufacturer's protocol. To check for purity and viability, the cells were stained with a PE-conjugated anti-CD34 antibody (555822; BD Biosciences) and DAPI, and then analyzed on a MACSQuant flow cytometer (Miltenyi Biotec, Inc.).

Loading of SF into cells

For single-cell experiments, SF was loaded into cells by incubating 5×10^5 cells in 100 μL culture media containing

freshly diluted SF for 30 min. SF concentrations of 20 and 80 μM were used for reporter loading in K562 cells and primary cells, respectively. Cells were stored at 37 °C in a 5 % carbon dioxide atmosphere during incubation with SF. Cells were pelleted and then washed 5× with 200 μL physiologic buffer (135 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 10 mM HEPES, and 10 mM glucose at pH 7.4). Cells were then resuspended in physiologic buffer at a concentration of 1×10^6 cells/mL and immediately loaded into the arrayed cell traps.

Measurements of SK activity in PBMC lysates

For ensemble measurements of SK activity, 5×10^5 PBMCs were pelleted and resuspended in culture media at a concentration of 5×10^6 cells/mL. The cells were then incubated with 80 μM SF for 1 h at 37 °C and 5 % carbon dioxide. During reporter incubation, cells were gently resuspended every 15 min to minimize settling. After 1 h, cells were pelleted, washed 5× with 200 μL physiologic buffer, and resuspended in 10 μL physiologic buffer. Cells were lysed by adding 10 μL methanol and stored at −80 °C until analysis. Lysate samples were diluted in electrophoretic buffer (27 mM monosodium phosphate and 10 % 1-propanol at pH 7.3) and analyzed using a custom-built CE system, described previously [33]. The sample was separated in a 35-cm fused-silica capillary with an inner diameter of 30 μm. The field strength was 450 V/cm, and laser-induced fluorescence detection was performed 4 cm from the capillary inlet. Standards of SF and S1PF were used to identify analyte migration times. Electropherograms were analyzed using a multiple peak fit function for Gaussian peaks (OriginPro 8.1, OriginLab, Northhampton, MA).

Capture of cells in three-channel device

Prior to the single-cell analysis, the three-channel device containing cell traps was mounted on an inverted microscope (Ti-E, Nikon (Melville, NY)) with a computer-controlled motorized stage (Ti-SER, Nikon). The stage was fitted with a temperature controlled stage insert, maintained at 37 °C for the duration of the experiment. The address of each cell trap in the microwell array was calculated using a customized Python program (Wolfeboro Falls, NH) [33]. Cells loaded with SF reporter were seeded into the microwells by placing 10 μL of a solution of cells on the microwell array. After allowing the cells to settle into the cell traps for 5–10 min, excess cells were rinsed from the array with physiologic buffer. The trapped cells were then continually bathed in physiologic buffer at 35 °C (1 mm/s flow velocity). Each cell trap with a single cell was manually identified and its address input into a customized program for subsequent analysis.

Single-cell analysis of SK activity with capillary electrophoresis

Automated single-cell analysis was performed as described previously [33]. Prior to analysis, the inlet of a 35-cm, 30 μm i.d. capillary was manually aligned 50 μm above the plane of the microwells. Upon the start of the program, the following events occurred sequentially under computer control. The microscope stage moved to the address of the first cell, placing the cell directly below the inlet of the capillary (Figure S1 Electronic Supplementary Material). The cell was lysed using a focused laser pulse [35] and 5 kV was applied across the capillary for 1 s to electrokinetically inject the cellular contents. The applied voltage was then set to zero for 1 s and the stage was moved to the center of the electrophoretic buffer channel. During this stage movement, the capillary was transferred through the air gap connecting the electrophoretic and physiologic buffer channel so that the alignment of the capillary was not disturbed. In addition, the air gap prevented the physiologic and electrophoretic buffers from mixing. Once the capillary was moved into the electrophoretic buffer, a voltage of 18 kV was applied across the capillary for 55, 56, or 57 s to separate the cellular contents. The voltage was then set to zero for 1 s during which time the capillary was moved back to the physiologic buffer and placed at the address of the next cell to be assayed. This process was repeated until all of the cells in the array were analyzed. A Python program controlled the motorized stage while all other tasks were performed using customized software written in LabVIEW (National Instruments, Austin, TX). Data was analyzed using a custom-built MATLAB program (Natick, MA) [36]. Cells with fluorescence values that saturated the PMT were omitted from subsequent analysis.

Results and discussion

Characterization of SK activity in tissue-cultured cells of leukemic origin

In order to characterize system performance using cells of leukemic origin, a tissue-cultured cell line, K562 (derived from CML), was initially analyzed. K562 cells were incubated with SF, washed, and trapped within arrayed microwells prior to analysis by automated CE [33]. The automated CE system was used to sequentially lyse, inject, and separate the contents of single K562 cells in the microwell array (Figure S1 Electronic Supplementary Material). Individual K562 cells were analyzed at a rate of 0.98±0.07 cell/min, in order to fully separate SF, S1PF, and additional fluorescent products without peak overlap from sequentially injected cells. Ninety-four cells were measured over the course of five experiments.

The resulting electropherograms contained as many as three fluorescent peaks (Fig. 1a). The first peak migrated at 33±2 s, a migration time identical to that of SF standards. The second peak co-migrated with standards of S1PF at 45±2 s. The final peak, termed U_{50}, was of unknown identity and migrated at 50 ±2 s. The separation efficiencies were 4,000±2,000, 8,000± 4,000, and 8,000±4,000 for SF, S1PF, and U_{50}, respectively. The resolution was 5.3±1.1 for SF and S1PF and 2.3±0.6 for S1PF and U_{50}. The automated CE system enabled fast and efficient separations on these nonadherent cells derived from leukemia.

In order to characterize SK activity in all of the analyzed K562 cells, a customized MATLAB program was developed to identify peaks attributable to each cell and measure their migration time, peak area, and full width at half maximum. The peak areas were then compared to that of standards of known concentration to estimate the number of moles of SF and S1PF found in each cell [36]. The number of moles of U_{50} was estimated by comparing the peak area of the U_{50} peak to the average moles per peak area of SF and S1PF standards. The majority of cells (79±30 %) formed S1PF, although in varying amounts (Fig. 1b). The percentage of reporter converted to S1PF ($S1PF_{mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol})$) varied from 0 to 100 % (Fig. 1c). Distinct subpopulations of cells were not readily identifiable with respect to the percentage of S1PF formed. The amount of reporter loaded into the cells varied by as much as four orders of magnitude, with an average of 6±14 amol per cell. The percentage phosphorylation of the reporter did not correlate with the total amount of reporter loaded into the cell (coefficient of determination (R^2)=0.03) or the time after the cell was loaded with SF (R^2=0.08) (Figure S2a Electronic Supplementary Material). Previous research has also indicated that the phosphorylation of the reporter was not dependent on the amount of reporter loading or the time after loading [33]. This likely indicates that the heterogeneity of single-cell SK activity obscures these trends.

When observed at the single-cell level, the metabolism of SF in K562 cells was extremely heterogeneous. The average percentage of the reporter that was phosphorylated in K562 cells was 39 %. However, only 18 % of cells possessed 34 to 44 % of the SF in the phospho form, and nearly as many cells (17 %) had either very high (>95 %) or low (<5 %) levels of phosphorylation. Single-cell measurements of SK activity provided a significantly richer picture of sphingosine metabolism than that derived from population-averaged data. The distribution of SK activity in the K562 cells was quite different from that previously measured in U937 cells, a histiocytic lymphoma/sarcoma-derived cell line with myeloid leukemia properties [33]. In the U937 cells, two cell populations were readily observed with respect to SK activity, a high state (>80 % of SF converted to S1PF) and a low state (<1 % of SF converted to S1PF). Most U937 cells (78 %) existed in the high state, whereas only 9 % of K562 cells possessed >80 % phosphorylated S1PF. Even though both cell lines were derived from aggressive hematologic malignancies, SK activity varied

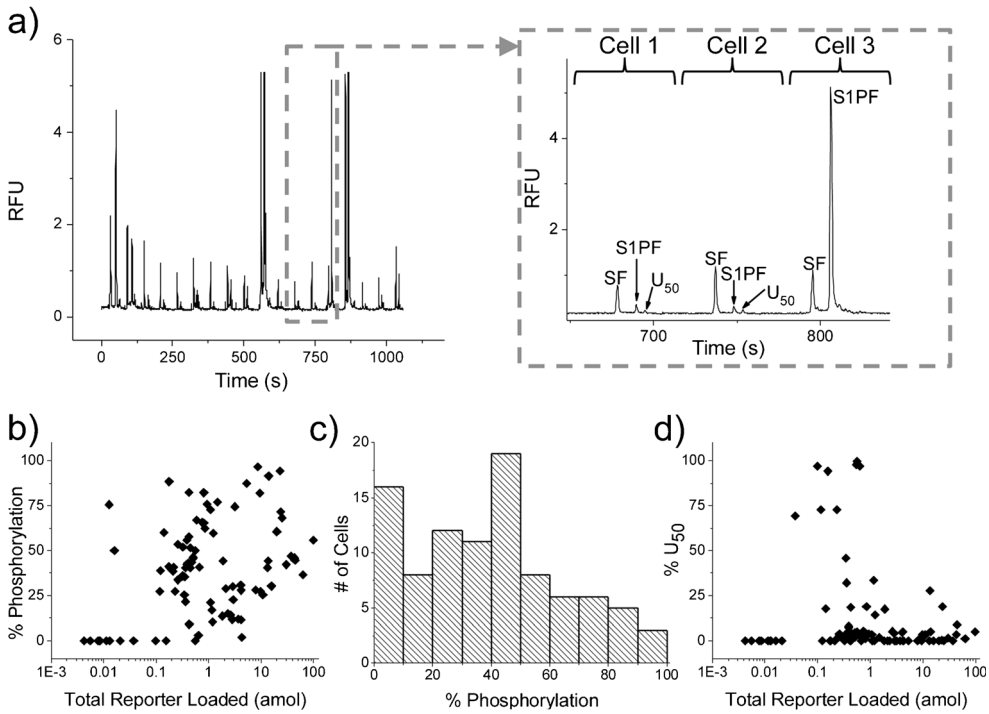

Fig. 1 SF reporter metabolism in single K562 cells. **a** A representative electropherogram of a single-cell experiment in which SF, S1PF, and U_{50} were separated in 18 K562 cells. (*inset*) An expanded region of the electropherogram shows the separation of the reporter and its metabolites in 3 cells. **b** Percent of phosphorylated reporter ($S1PF_{mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol})$) plotted against the total amount of reporter loaded ($SF_{mol}+S1PF_{mol}+U_{50\ mol}$) into each cell ($n$=94). **c** The distribution in the percentage of SF phosphorylated in K562 cells. **d** The percentage of SF converted to U_{50} ($U_{50\ mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol})$) plotted against the total reporter loaded into a cell

greatly between these two cell types. Both cell types did possess cells expected to be susceptible to SK inhibitors as well as unresponsive to SK inhibitors.

The percentage of U_{50} formed in single K562 cells varied across the cell population by as much as that for S1PF. Sixty percent of K562 cells converted SF to U_{50} and within this population there was significant heterogeneity in the percent of the reporter that was converted (Fig. 1d). The average percentage of U_{50} formed with respect to total reporter for all K562 cells was 12±25 %. Since U_{50} was not observed in cells that were not loaded with SF, U_{50} was most likely formed from an unidentified enzyme acting on SF or a downstream metabolite of the SK pathway (Figure S3 Electronic Supplementary Material). The identification of U_{50} awaits synthesis of standards of additional fluorescent metabolites of the sphingosine signaling pathway. The number of moles of U_{50} produced in cells was weakly correlated with S1PF (R^2=0.46), the total amount of SF loaded into the cell (R^2=0.26) and the incubation time of the reporter in the cell (R^2=0.22) (Figure S2b–d Electronic Supplementary Material). The amount of U_{50} produced in cells was more strongly correlated with the amount of S1PF formed rather than the total SF loaded into the cell suggesting that U_{50} might be a downstream product of S1PF, such as hexadecenal fluorescein (Figure S3 Electronic Supplementary Material). The migration time of U_{50} also suggested that it possessed a more polar chemical structure than SF, which additionally supports the hypothesis that it might be a downstream product of S1PF.

Analysis of primary peripheral blood mononuclear cell populations

To evaluate the activity of SK in human primary cells, initial experiments focused on the suitability of freshly frozen PBMCs from leukemic patients. These PBMCs contain a mixed population of mononuclear blood cells, including immature leukemic blasts as well as more differentiated tumor cells and normal lymphocytes, monocytes, and macrophages. Frozen PBMCs were chosen as a tissue source due to the ready availability of frozen specimens. However, frozen primary PBMCs can rapidly lose viability upon thawing [37]. Consequently, initial experiments focused on identifying the time span over which the cells remained intact as determined by trypan blue exclusion assays. Immediately upon thawing, 94±2 % of cells excluded trypan blue but this number dropped to 87 ±2 % after 4 h and to 71±1 % after 24 h. The number of cells in the culture volume also decreased by 69±16 % over the first 24 h. These data suggested that cells should be assayed immediately after thawing to maximize viability.

While the frozen primary cells might be viable immediately after thawing, their rapid decline over 24 h suggested that the cells may be on a programmed pathway to death and not suitable for use in signaling assays, particularly assays investigating apoptosis. To determine whether SK activity was altered by freezing, SK activity was measured in cell lysates prepared from fresh or thawed cells from the same leukemic patient. PBMCs were collected from a patient, and divided into two samples. The first sample was incubated with SF, lysed, and then assayed for formation of S1PF. The second sample was frozen for 24 h, thawed, and then immediately incubated with SF and lysed. The lysate prepared from frozen

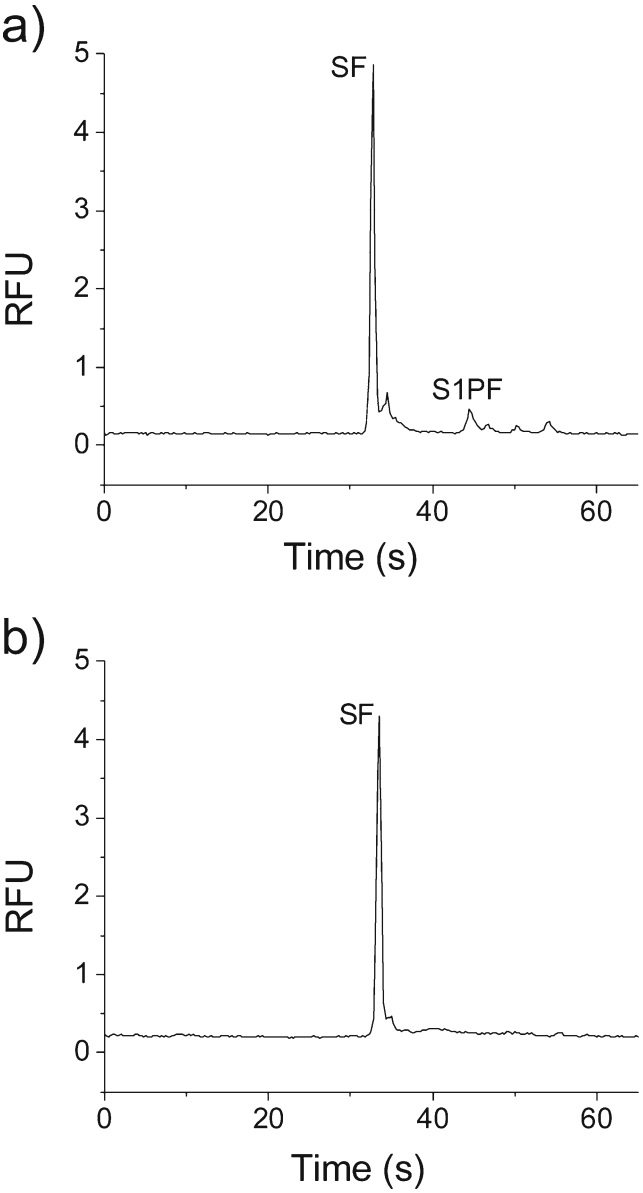

Fig. 2 Comparison of SF reporter conversion in fresh vs frozen PBMC lysates. **a** An electropherogram showing the conversion of the SF reporter to S1PF and several unidentified metabolites in a lysate prepared from fresh PBMCs. **b** An electropherogram demonstrating the decrease in SF metabolism in a lysate prepared from frozen PBMCs

cells exhibited decreased conversion of SF to S1PF relative to the lysate from fresh cells (0 vs 10 % of SF converted to S1PF) (Fig. 2a, b). The freshly prepared lysate also exhibited a number of SF metabolite peaks that were not present on the electropherograms obtained from the previously frozen cell lysate. Similar results were obtained for K562 cell lysates, in which conversion of the SF reporter to S1PF and additional metabolites decreased by 46±24 % after being frozen and thawed. These data suggest that frozen primary cells (and frozen tissue-cultured cells) rapidly lose SK activity as well as other related enzymatic activities upon thawing and are not suitable for assay of the SK pathway. Therefore, fresh primary cells were used for all subsequent assays.

Measuring single-cell SK activity in PBMCs of patients with leukemia

In order to characterize the activity of SK in primary samples, PBMCs of leukemic patients were isolated from a patient with accelerated phase CML with 5 % leukemic blasts (the other white blood cells were leukemia-derived but more differentiated), a patient with relapsed AML with 87 % blasts, and a patient with de novo B-ALL with 20 % blasts. SK activity was then measured in freshly isolated cells loaded with SF and without regard for the cell type within the sample. A total of 22, 13, and 39 cells from a single CML patient, a single AML patient, and a single B-ALL patient were analyzed, respectively. Due to the heterogeneity of the samples, it was expected that 1, 11, and 8 of these cells were leukemic blasts, for CML, AML, and B-ALL PBMC samples, respectively. Single-cell traces from each patient are shown in Fig. 3a–c. All PBMC samples possessed cells with analytes that co-migrated with SF and S1PF. Two cells from the CML patient and three cells from the AML patient displayed up to five unidentified peaks that were named based on their migration times, U_{50} (50.6±0.1 s), U_{53} (53.0±0.2 s), U_{55} (54.8±0.1 s), U_{57} (57.5±0.5 s), and U_{60} (60.9±0.6 s) (Fig. 3a). The number of peaks per cell varied within each patient, as well as between patients (Fig. 3d). The total amount of reporter per cell varied from 1.7±2 amol to 21±80 amol for the patient samples, but was not significantly different between the three patient samples (Table 1). These levels are similar to endogenous amounts of sphingosine, which ranges from 5–30 amol per cell [38, 39]. The percentage of SF converted to S1PF varied considerably among the cells from all three patients (Fig. 3e). However, there were too few cells to accurately measure the correlation between the amount of S1PF and the reporter loading amount and time (Figure S4 and S5 Electronic Supplementary Material). In contrast to U937 and K562 cells, the majority of PBMCs converted very little SF to S1PF (Fig. 3f). None of the patients had significantly different levels of phosphorylation or production of unidentified compounds despite the diverse disease states and dissimilar numbers of normal vs tumor cells. These samples demonstrated the

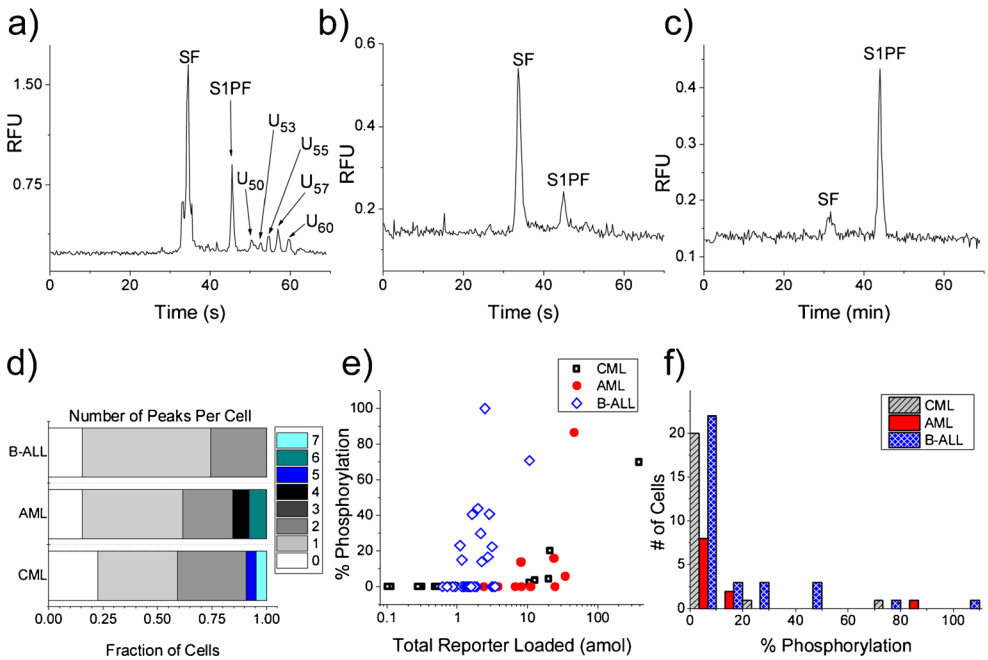

Fig. 3 Measurements of reporter metabolism in single PBMCs from three patients with different types of leukemia. **a**–**c** Electrophoretic traces from a CML patient with 5 % leukemic blasts (**a**), an AML patient with 87 % leukemic blasts (**b**), and a B-ALL patient with 20 % leukemic blasts (**c**). **d** The distribution of the number of electropherogram peaks identified in each cell for the different patients. **e** The percent of reporter phosphorylation ($S1PF_{mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol}+U_{53\ mol}+U_{55\ mol}+U_{57\ mol}+U_{60\ mol})$) plotted against the total amount of reporter loaded into each cell ($SF_{mol}+S1PF_{mol}+U_{50\ mol}+U_{53\ mol}+U_{55\ mol}+U_{57\ mol}+U_{60\ mol}$) for each patient. **f** A histogram showing the percentage of S1PF relative to total SF loaded in the PBMCs for each patient

Table 1 Properties of PBMCs and $CD34^+$ cells for the four leukemic patients

Parameter averages (per cell)						
Cells	% Blasts	SF loaded (amol)	Incubation time (min)	% S1PF	% U_{50}	Total number of cells assayed
CML patient PBMCs	5	21±80	171±7	16±25	0.8±2.5	22
AML patient PBMCs	87	13±20	195±4	11±26	0.8±2.5	13
B-ALL patient PBMCs	20	1.7±2	185±20	13±23	0±0	39
AML $CD34^+$	99	0.12±0.2**	92±20**	19±36	27±39*	45

*$p<0.05$; **$p<0.01$

p values were calculated by comparing all cellular measurements per patient between all four patients

challenge of performing measurements on patient samples comprised of very small numbers of mixed cell types. A future step for this work will be to combine single-cell CE with fluorescence microscopy of immunostained cells to distinguish cell types just prior to CE analysis. However, these data do demonstrate the ability of the automated CE system to detect fluorescent sphingosine and its metabolites in single primary cells.

Single-cell SK activity in isolated $CD34^+$ AML blasts

In order to characterize SK activity in leukemic blasts, $CD34^+$ leukemic cells were isolated from an AML patient. CD34 is a leukemic stem cell marker expressed in a subset of AML patients and CD34 expression is linked to poor prognosis [40]. An AML patient expressing CD34 was identified by flow cytometry of the peripheral blood cells and the $CD34^+$ cells were then enriched by positive selection using magnetic-activated cell sorting (Fig. 4a). The enriched cells were then examined by flow cytometry for viability and purity (Fig. 4b). Ninety-two percent of the cells were judged to be viable using DAPI exclusion, and 99 % of the viable cells expressed CD34. SK activity was then measured in this purified population of freshly isolated $CD34^+$ leukemic cells.

The $CD34^+$ leukemic stem cells differed from the mixed PBMC populations by several attributes. The $CD34^+$ cells possessed a ten-fold lower amount of SF relative to the prior samples despite being loaded with SF under identical conditions (Table 1). $CD34^+$ cells are known to express high levels of drug efflux pumps, so the lower amount of SF measured in $CD34^+$ cells may be due to higher levels of SF efflux [41, 42]. Despite containing less SF, the average percent phosphorylation per cell was 19±36 %, which was comparable to the PBMCs. A total of 18 % of cells phosphorylated the reporter, at levels from 45 to 100 % phosphorylation (Fig. 4c). The percentage of SF converted to S1PF did not correlate with the incubation time for SF within the cell (R^2=0.02) and was not strongly correlated with total SF loaded into the cell (R^2= 0.34) (Figure S6 Electronic Supplementary Material). SK activity was highly variable when viewed at the single cell

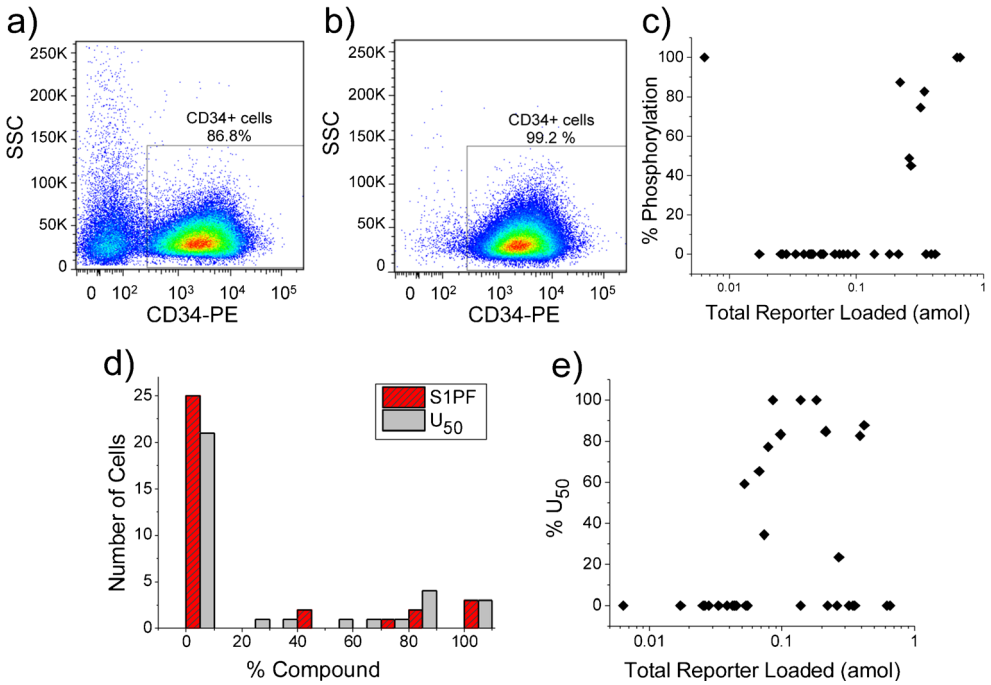

Fig. 4 Single-cell measurements in $CD34^+$ cells isolated from the PBMCs of an AML patient. **a** Flow cytometry data of the PBMCs prior to $CD34^+$ enrichment by MACS. Side light scatter (SSC) is plotted against phycoerythrin (PE) fluorescence. The cells were labeled with PE-α-$CD34^+$. **b** Flow cytometry data of the MACS-enriched $CD34^+$ PBMCs. **c** Percent of phosphorylated reporter ($S1PF_{mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol})$) plotted against the total amount of reporter loaded into a $CD34^+$ cell ($SF_{mol}+S1PF_{mol}+U_{50\ mol}$). **d** Histogram showing the distribution of reporter phosphorylation and conversion to U_{50} ($U_{50\ mol}/(SF_{mol}+S1PF_{mol}+U_{50\ mol})$). **e** The percent of reporter converted to U_{50} was plotted against the total reporter loaded into a cell

level even in this relatively pure population of cells from a single patient. Moreover, a minor subpopulation of the cells displayed very high SK activity relative to the other cells in the population. These results support previous findings that there is immense phenotypic heterogeneity in leukemia [43], and demonstrate that understanding the diversity of SK signaling in single primary cells will be important to determine the suitability of SK inhibitors as treatments for leukemia.

In addition to displaying heterogeneous SK activity, the $CD34^+$ cells also demonstrated variable conversion of the reporter to the unidentified peak U_{50}. Twenty-seven percent of $CD34^+$ cells contained U_{50}, at levels ranging from 23 to 100 % of the reporter present (Fig. 4d–e). The $CD34^+$ cells possessed a significantly higher average percentage of U_{50} per cell than the mixed PBMCs (Table 1). Interestingly, there was a greater average percentage of reporter converted to U_{50} (27± 39 %) in the $CD34^+$ cells than that for S1PF in any of the primary cells examined. It will be important to identify U_{50} in future studies to understand whether it is a metabolite of S1PF, in which case SK inhibitors might still be effective against the U_{50}-generating cells. If U_{50} is a product of enzymes upstream of SK, however, then SK inhibitors would likely not be effective in a patient such as this (Figure S3 Electronic Supplementary Material). This work, in addition to existing studies [44], demonstrates the necessity of fully characterizing multiple signaling pathways at the single-cell level in order to identify the best therapeutic targets in individual patients.

Conclusion

Measurement of SK activity in primary and immortalized cells of the hematopoetic system was optimized. Frozen cells were found to be inferior to fresh samples because freezing and thawing resulted in a significant drop in SK activity as well as the activity of other enzymes within this pathway. Thus, fresh samples are critically important for accurate measurements of SK signaling. SK activity was highly variable within single cells from freshly isolated PBMCs. Since mixed populations of cells were present in each PBMC sample, the heterogeneity may have reflected the diverse cell types within the sample. However, when SK activity was measured in a nearly pure population of leukemic blasts, these cells also demonstrated a significant level of heterogeneity in SK activity. The SK pathway was highly active in a subpopulation of these $CD34^+$ blasts cells; however, the majority of the cells did not convert substantial quantities of SF to other metabolites in the SK pathway. These blasts also generated significantly more of the unidentified compound U_{50} than the tissue-cultured cells or the PBMCs. A future goal of this work will be identification of the U_{50} compound and consequently the enzyme(s) responsible for converting SF into this metabolite. This may enable identification of additional drug targets in leukemic cells.

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