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Evaluation of Anticancer Agents Using Flow Cytometry Analysis of Cancer Stem Cells

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Abstract

Flow cytometry can sensitively detect and efficiently sort cells based on fluorescent signals integrated into cellular markers of proteins or DNA. It has been broadly applied to assess cell division, apoptosis and to isolate cells including stem cells. As the seeds for tumorigenesis and metastasis, cancer stem cells (CSCs) are often more resistant to cytotoxins and anticancer agents than other heterogeneous cells in tumors. Analyzing CSCs under treatments is an effective way to evaluate new therapeutic agents for cancers. We introduce a method using flow cytometry to assess breast CSCs (CD44⁺/CD24^{-/low}) in human MCF-7/Dox breast cancer cells, after the treatment of mixed-backbone oligonucleotide against glucosylceramide synthase. Flow cytometry analysis of CSCs is a reliable, effective, and easy-handling approach to screen agents targeting CSCs.

Keywords

Cancer stem cells; CD44; CD2; Flow cytometry; Glucosylceramide synthase; Mixed-backbone oligonucleotide

1. Introduction

Tumorigenesis and cancer progression depend on a small subset of cancer cells known as cancer stem cells (CSCs). In addition to initiating primary tumors, CSCs are the seeds of tumor metastasis and relapse after clinical remission (1, 2). Besides sharing the same properties of normal adult stem cells in self-renewal and generation of diverse cells, CSCs display cancerous characteristics and are resistant to cytotoxins (3). In human breast cancer, the cells with CD44⁺/CD24^{-/low} markers are identified as breast cancer stem cells (BCSCs) (4). These cells are more resistant to anticancer agents and radiation than other heterogeneous cells in tumors (5, 6). Thus, the significant drug resistance with cancerous stemness earmarks CSCs as novel targets for cancer treatments (7, 8).

Analysis of CSCs under treatments becomes a more effective way to evaluate new therapeutic agents for cancers. Here, we describe a method using flow cytometry to assess BCSCs in an established cell line after the treatment of mixed-backbone oligonucleotide (MBO-asGCS) against glucosylceramide synthase (GCS) (9). Based on specific stem cell markers, such as CD44⁺/CD24^{-/low}, flow cytometry can quantitatively determine the alterations of BCSCs after treatments (4, 5, 10). Established cancer cell lines that are enriched with CSCs have been broadly used as sources to study the properties of stem cells (5, 11). Human MCF-7/Dox breast cancer cell line has greater than 20% of side-population cells or CD44⁺/CD24^{-/low} cells as we assessed. MCF-7/Dox cells grow well in standard

culture condition. The use of MCF-7/Dox cells, rather than the isolated BCSCs, allows directly assessing the effects of agents on BCSCs in a simple procedure.

We use MBO-asGCS as an example of agents that disrupt CSCs. GCS catalyzes the first glycosylation of ceramide in glycosphingolipid synthesis and has a vital role in mouse embryo development (12, 13). Enhanced capacity of ceramide glycosylation allows human embryonic stem cells growth (14). Our works suggest that MBO-asGCS can effectively disrupt BCSCs (unpublished data). MBO-asGCS treatment significantly decreases BCSCs (13.84 vs. 20.59) detected by flow cytometry analysis and immunostaining of CD44/CD24.

Taken together, the flow cytometry analysis of CSCs presented is a reliable, effective, and easy-handling approach to screen new agent to target CSCs.

2. Materials

1. Drug-resistant MCF-7/Dox human breast cancer cells (University of Texas, M.D. Anderson Cancer Center, Houston, TX). MCF-7/Dox cells were derived from the parental drug-sensitive MCF-7 human breast cancer cells by stepwise selection with doxorubicin (15).
2. RPMI-1640 medium (Invitrogen, Carlsbad, CA): supplemented with 10% fetus bovine serum (FBS) (Hyclone, Logan UT), 100 U/mL penicillin, 100 µg/mL streptomycin, and 584 mg/L l-glutamine.
3. Phosphate-Buffered Saline (PBS): pH 7.4 prepared by 10 times dilution of PBS (10×) with distilled water following autoclave sterilization.
4. 0.25% Trypsin with EDTA·4Na (Invitrogen).
5. Lipofectamine™ 2000 (Invitrogen).
6. Opti-MEM I reduced-serum medium (Invitrogen).
7. An MBO was designed to target the ORF 18–37 of human GCS and designated as MBO-asGCS. A scrambled control had the same chemical components as MBO-asGCS, but no sequence specificity designated as MBO-SC. MBOs were purified by reverse-phase HPLC and desalting after synthesis (Integrated DNA Technologies, Inc., Coralville, IA) (9, 16). MBOs were dissolved in sterile water in 150 µM and stored at −80°C after aliquot.
8. Blocking buffer: 5% goat serum in PBS.
9. FITC anti-human CD44 antibody (BioLegend, San Diego, CA) (25 µg in 100 µL); Alexa Fluor® 647 anti-mouse CD24 antibody (Cat. 101817, BioLegend) (25 µg in 100 µL).
10. Polystyrene round bottom test tubes (5 mL, 12 × 75 mm) (BD Bioscience, San Jose, CA) are used as the flow-cytometer tube.
11. Sheath Fluid (BD Bioscience).
12. BD FACSCalibur™ Flow Cytometer equipped with lasers argon (L1) and red diode (L2) (BD Bioscience).
13. Software in BD FACSCalibur: BD CellQuest Pro (BD Bioscience).
14. BD Falcon 4-well CultureSlide (BD Bioscience).
15. Mouse anti-human CD44 monoclonal antibody (Cat. C7923, Sigma-Aldrich) (200 µg/mL).

16. Rabbit anti-human CD24 polyclonal antibody (FL-80) (Cat. SC-11406, Santa Cruz Biotechnology, Santa Cruz, CA) (200 µg/mL).
17. Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (2 mg/mL in 0.5 mL) (Cat. A-11008, Invitrogen).
18. Alexa Fluor® 555 goat anti-mouse IgG (H+L) (2 mg/mL in 0.5 mL) (Cat. A-21422, Invitrogen).
19. VECTASHIELD Mounting Medium with DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories Inc., Burlingame, CA).

3. Methods

3.1. Cells Treated with MBO-asGCS

1. Seed MCF-7/Dox cells ($\sim 2 \times 10^6$ cells) in 100 mm tissue culture dishes and add 10 mL of 10% FBS RPMI 1640 medium per dish. incubator until the cells
2. Culture the cells at 37°C in a CO₂ achieve 70–80% confluence. This usually takes 18–24 h.
3. Prepare transfection solution in two 15-mL sterile test tubes:
 Solution A: add 8 µL of MBO-asGCS stock solution (150 µM) into 3 mL Opti-MEM I medium, to prepare 3 mL of 200 nM MBO-asGCS solution per dish.
 Solution B: add 20 µL of Lipofectamine 2000 into 3 mL Opti-MEM I medium to prepare 3 mL liposome solution per dish.
4. Gently shake the Solutions A and Solution B and stand them still at room temperature ($\sim 26^\circ\text{C}$) for 5 min.
5. Gently mix Solution A and B together in one tube and stand it still at room temperature for 20 min to form MBO-liposome complex. For Vehicle control, Solution B is mixed with additional 3 mL Opti-MEM I medium.
6. During this period (see step 5 above), remove the medium from the cell dishes (see step 2 in the Subheading 3.1) and rinse the cells once with 10 mL of sterile PBS.
7. Slowly drop the transfection solutions (see step 5 in Subheading 3.1) into cell dishes with gently shaking. Experimental dishes are transfected with MBO-asGCS/ Lipofectamine, and the Vehicle dish is mock-transfected with Lipofectamine only.
8. Incubate the cells in dishes at 37°C for 5 h in a CO₂ incubator.
9. Add 20% FBS RPMI 1640 medium, 6 mL per dish. The final concentration of FBS will be 10% in medium with 100 U/mL penicillin, 100-µg/mL streptomycin, and 584 mg/L l-glutamine.
10. Continuously culture cells for an additional 48 h, and then analyze the cells using flow cytometry and immunostaining.

3.2. Sample Preparation

1. Remove medium from the cell dishes (see step 10 in Subheading 3.1), and detach cells by spreading 500 µL of 0.25% trypsin-EDTA to the dishes.
2. Add 10 mL PBS to collect all cells from each dish and spin down cells in 15-mL tubes, 720 rcf, and at room temperature for 5 min.

3. Resuspend the cell pellets in PBS at a density of 10^6 cells/100 μ L. Prepare two vials (10^6 cells/100 μ L in 1.5-mL Eppendorf tube) for each sample.
4. Add 5 μ L of FITC-CD44 and 5 μ L of Alexa647-CD24 antibodies into each cell tube marked the Stained. Add 10 μ L of PBS into the Unstained tubes.
5. Incubate the cells with the fluorescent antibodies for 30 min at 4°C in refrigerator. Cells are covered with aluminum foil to prevent fluorescence quenching (see Note 1).
6. Spin down the cells at 720 rcf, room temperature for 10 min. Resuspend the cell pellets in 1 mL of PBS and spin down the cells.
7. Resuspend the cell pellets in 1 mL of PBS and transfer into the flow-cytometer tubes on ice and cover with aluminum foil. These samples including the Unstained Vehicle, Vehicle, Unstained MBO-SC, MBO-SC, Unstained MBO-asGCS, and MBO-asGCS are ready for flow cytometer analysis (see Note 2).

3.3. Analysis of BCSCs by Flow Cytometer

3.3.1. Start-Up Flow Cytometer

1. Open the front lid of the flow cytometer (BD FACSCalibur), fill the sheath fluid, and empty the waste tank.
2. Turn on the cytometer, and then turn on the computer.
3. In the front lid of cytometer, turn switch from the Change Tank position to the Pressurize position.

3.3.2. Create Experimental Windows

1. Open the CellQuest Pro program from the computer. An untitled document with Tool Palette will automatically appear. The following steps will create an experimental window (Fig. 1).
2. Click Acquire from the Menu bar, select Connect to Cytometer. A Browser Window will appear. This step connects cytometer to computer.
3. Click Cytometer followed by selecting Detector/Amps from the Menu bar. In the Detector/Amps window, check Four Color and select FL2 for DDM Param. This step will allow FL4 Detector showing in the Detectors/Amps window.
4. Click the Dot Plot icon from the Tool Palette or click Plots followed by selecting Dot Plot from the Menu bar. Customize the plot size in the untitled document. In this step, an Inspector:Dot Plot window will automatically appear. In this window, change Analysis to Acquisition position. Select FSC-H 1024 and Lin for X Parameter and SSC-H 1024 and Log for Y Parameter in the Inspector:Dot Plot window. Thus, the FSC on X-axis and SSC on Y-axis (FSC vs. SSC) will be shown in the dot plot window. This dot plot will be used to detect all cells.
5. Repeat step 4 to establish second dot plot for detecting two colors of fluorescence (green and red). Select FL1 1024 for green color (FITC) and FL4 1024 for red color (Alexa Fluor® 647) in the Inspector:Dot Plot window. The dot plot window

¹Fluorescence antibodies are susceptible to light, it is necessary to avoid the exposure to light. Once the fluorescent antibodies are applied to the cells, use aluminum foil to cover the tubes or slides.

²Immediately analyze cell samples. Once the antibody incubation is completed, it is always advisable to start the flow cytometer assay.

will show the Alexa Fluor[®] 647-CD24 on X-axis and FITC-CD44 on Y-axis (FL4 vs. FL1).

6. Click Cytometer from the Menu bar, and select Detector/Amps, Threshold, Status, and Counters separately. This will allow appearing four different windows. These parameters include detector/amps need to be adjusted while analyzing the samples. Keep all these windows aside the experiment document.
7. Click Acquire from the Menu bar and select Acquisition. The storage of the Acquisition and storage dialog box will appear. In the box, select acquisition gate accept, all events, collection criteria 10,000 of all events are counted and storage gate all events, and then click on OK (Fig. 1).
8. Choose Acquire from the Menu bar and select Parameter Description and a new window will appear. In the window, select change Directory button, create a new folder as “BCSC,” and provide the location for this folder. Select change File, a window of File Name Editor will appear. In Custom Prefix, provide the file name as “MCF-7/DOX.” The File Name Prefix will be Custom Prefix and File Name Suffix will be File Count. In the File Count put “1” and click on OK. Once data acquisition starts, the CellQuest Pro will automatically save the data as “MCF-7/DOX 1” (FCS file) in the folder of “BCSC.”

3.3.3. Data Acquisition

1. From the Tool Palette, select the quadrant marker and divide the second dot plot (FL4 vs. FL1) in four quadrants (Fig. 2).
 - Q1 Lower left quadrant (LL), showing both negative cells
 - Q2 Upper left quadrant (UL), showing green-positive cells
 - Q3 Upper right quadrant (UR), showing both positive cells
 - Q4 Lower right quadrant (LR), showing red-positive cells
2. Adjust the parameters (Voltage and Amp Gain in the Detector/Amps window) while analyzing the Unstained cells. The same parameters should be used for the stained cells. These parameters should be adjusted every time when analyzing different types of cells or cells treated in different conditions (see Notes 3 & 4).
3. Put the unstained sample, “unstained vehicle” (see step 7 in Subheading 3.2) in the Sample port of the cytometer (see Note 3).
4. Change the cytometer in Run mode, and Speed Low.
5. Go into the Acquisition Control window and check for the Setup box.
6. Click on the Acquire now. This will allow us to view samples in all the plots and adjust the instrument settings.
7. While looking at the sample in the first dot plot (FSC vs. SSC), adjust the Voltage and Amp Gain in the Detector/Amps window to make sure all events are in this plot. Then, looking at the second dot plot (FL4 vs. FL1), adjust the Voltage and the

³It is necessary to vortex sample just before placing it to the Sample Port. It can decrease cell clumps and avoid block of the Sample Port.

⁴Adjust the parameters with unstained cells every time, before you start to acquire data for different cell line or cells treated with different conditions. Each cell line or treatment has its own cell sizes and internal complexity that determine the cell signals detected by cytometer.

Amp Gain from the Detector/Amps window, until entire events in the first quadrant Q1 (Fig. 2).

8. Look in the contour plots, and make sure that peak in the contour plots with the filter FL1 and the FL4 in the left. Otherwise, adjust the Voltage in the Detector/Amps to position the peak in the left side of the contour plots.
9. After necessary changes in the instrument settings, click on the Pause and then the Abort in the Acquisition Control window.
10. Uncheck the Setup box, file name “MCF-7/Dox 1” will appear in the Acquisition Control window. Click on the Acquire button in the Acquisition Control.
11. Once cytometer finishes counting of the 10,000 events, the CellQuest Pro will automatically save the counting data of “unstained vehicle” in the file “MCF-7/Dox 1” and the “BCSC” folder. For the Unstained samples, these cells should be in the lower left quadrant, Q1.
12. The file named “MCF-7/Dox 2” for next sample will automatically appear in the Acquisition Control window, once cytometer finishes last counting. It is necessary to analyze the stained sample using the same instrument settings as unstained one. Put the Stained sample, “vehicle” in the Sample port (see step 7 in Subheading 3.2) and repeat the steps 10 and 11 in Subheading 3.3.3 to acquire the data for the Vehicle. The counting data for the “vehicle” will be automatically saved as “MCF-7/Dox 2” (see Notes 5 & 6).
13. Repeat the steps 3–11 in Subheading 3.3.3 with the sample of “unstained MBO-SC” to acquire the data for the “unstained MBO-SC.” The counting data for the “unstained MBO-SC” will be automatically saved as “MCF-7/Dox 3.”
14. Repeat the steps 3–11 in Subheading 3.3.3 to acquire the data for the “MBO-SC.” The counting data for the “MBO-SC” will be automatically saved as “MCF-7/Dox 4.”
15. Repeat the steps 3–11 in Subheading 3.3.3 with the sample of “unstained MBO-SC” to acquire the data for the “unstained MBO-SC.” The counting data for the “unstained MBO-SC” will be automatically saved as “MCF-7/Dox 5.”
16. Repeat the steps 3–11 in Subheading 3.3.3 to acquire the data for the “MBO-SC.” The counting data for the “MBO-SC” will be automatically saved as “MCF-7/Dox 6” (see Note 7).

3.3.4. Data Analysis

1. Choose plot from the Menu and select Dot Plot. This will open the Plot Inspector window. In the Plot Inspector window, click on the Select File and choose the file name (MCF-7/Dox 1) for analysis.
2. Once the data appear on the dot plot, select the FL4 on X-axis and the FL1 on Y-axis.

⁵It is necessary to measure the BCSCs for each sample in triplicate. For data acquisition, apply the same parameters and the same tube of sample, and repeat the Subheading 3.3.3, step 12 two times to acquire counting data for the stained sample. In data analysis, repeat the Subheading 3.3.4, step 6 two times to generate the data for the Region Stats for the triplet.

⁶For many groups of samples, note down the order of data acquisition to prevent confusion. The CellQuest Pro program saves the files under the same name plus counting order, such as “MCF-7/Dox 1,” “MCF-7/Dox 2,” and “MCF-7/Dox 3” in the recent analysis.

⁷Once the sample is analyzed (see step 11 in Subheading 3.3.3), always put the cytometer on the Stand-by mode to save the laser use.

3. From the Tool Palette, select the Quadrant icon, divide the plot into four quadrants and adjust dividing lines to locate the unstained cell sample in the both negative quadrant (Q1) (Fig. 3a, b).
4. In the Pool Palette, select the Rectangular icon, place the cursor in the desired quadrant, and create a region around that quadrant. To deselect a region, click on the region in the plot and the Delete in the Menu bar.
5. In the Stats, select the Region Stats in the Menu. A Region Stats window will appear, and it will provide us the events in desired region from the total 10,000 events.
6. Repeat the steps 1–5 in Subheading 3.3.4 with the file of “MCF-7/Dox 2” to generate the dot plot for the “vehicle.” The same fashion of quadrants and the desired region generated from the sample of “unstained vehicle” should be used for the “vehicle.” The cells in the rectangular region of the Quadrants 2 (Q2) are designated as BCSCs and the percentage of these cells after reduced the Unstained (see step 5 in Subheading 3.3.4) is presented with the dot plot (Fig. 3c).
7. Repeat the steps 1–6 in Subheading 3.3.4 with the files of “MCF-7/Dox 3” and “MCF-7/Dox 4” to create the dot plots for the “unstained MBO-SC” and “MBO-SC” (Figs. 3c, d).
8. Repeat the steps 1–6 in Subheading 3.3.4 with the files of “MCF-7/Dox 5” and “MCF-7/Dox 6” to create the dot plots for the “unstained MBO-asGCS” and “MBO-asGCS” (Fig. 3e, f).

3.4. Analysis of BCSCs by Immunostaining

1. Seed the cells (25,000 cell/chamber) in four-chamber slide and add 0.4 mL of 10% FBS RPMI 1640 medium and culture cells for 24 h.
2. Flip the medium out, and rinse the cells with 400 μ L of PBS.
3. After rinsing, fix the cells with 400 μ L of ice-cold methanol at 4°C for 5 min.
4. Flip the methanol out and keep the chamber upside down on paper towel and dry it in air for 5 min.
5. Rinse the chambers with 400 μ L of PBS, 5 min for 3 times, to rehydrate the cells.
6. Add 400 μ L of blocking solution (5% goat serum in PBS) and incubate at room temperature for 30 min.
7. Flip the blocking solution out and add 250 μ L of primary antibody solution. Mouse anti-human CD44 and rabbit antihuman CD24 antibodies are diluted with the blocking solution in 1:100.
8. Keep the slide on wet paper towel in plastic box to incubate at 4°C in refrigerator, overnight.
9. Remove the antibody solution and wash the chambers with 400 μ L of PBS for 5 min, 3 times.
10. Add 250 μ L of the secondary antibody solution into the chamber slides. The Alexa Fluo® 488 goat anti-rabbit IgG (H+L) (for CD24) and Alexa Fluor® 555 goat anti-mouse IgG (H+L) (for CD44) are diluted with the blocking solution in 1:1,000.
11. Incubate slides covered with aluminum foil at room temperature for 1 h (see Note 1).

12. Remove the second antibody solution and wash the slides with 400 μ L of PBS for 5 min and 3 times.
13. Remove the chambers from slide with the Remove Tool and observe the slides under fluorescent microscope to visualize the slide background.
14. Drop the VECTASHIELD Mounting Medium to cover cell section. This is for nucleus-counter staining and preventing fluorescence quenching. Use the slips to cover slides.
15. Observe slides under Olympus IX71 microscope coupled with digital camera (DP71). Images are captured using Olympus DP controller software (Fig. 4).

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Fig. 1. Analysis parameters in the CellQuest program. A typical experiment of flow cytometry assay in the dot and contour plots is settled with parameters in the detector/amps, threshold, counters, and acquisition control windows.

Fig. 2.

A dot plot for double-fluorescence cell assay. In the dot plot, the X-axis and the Y-axis represent the FL4 (red, Alexa647) and the FL1 (green, FITC), respectively. The dot plot is divided into four quadrants (Q1, Q2, Q3, and Q4). The quadrant Q1 will have the green-negative and red-negative cells, Q3 will have the green-positive and red-positive cells. The Q2, the green-positive and red-negative cells; the Q4, the green-negative and red-positive cells.

Fig. 3.

Effects of MBO-asGCS on BCSCs ($CD44^{+}/CD24^{-/low}$). MCF-7/Dox cells were exposed to MBO-asGCS or MBO-SC (200 nM) for 48 h and incubated with FITC-CD44 and Alexa647-CD24 antibodies. (a) Unstained cells for the vehicle. Cells cultured in the absence of MBOs (lipofectamine, vehicle) were used to settle the parameters for cell assay, and all the cells are in the quadrant 1 (Q1). (b) Stained vehicle. Cells cultured in the absence of MBOs (lipofectamine, vehicle) were incubated with FITC-CD44 and Alexa647-CD24 antibodies. The BCSC population (Q2) is $20.59 \pm 1.92\%$, from triplicate assay. (c) Unstained cells for MBO-SC treatment. Cells treated with MBO-SC were used to settle the parameters, and all the cells are in quadrant 1 (Q1). (d) Stained cells for MBO-SC treatment. Cells treated with MBO-SC were incubated with FITC-CD24 and Alexa647-CD44 antibodies. The BCSC population in the quadrant 2 (Q2) is $20.69 \pm 1.44\%$, from triplicate assay. (e) Unstained cells for MBO-asGCS treatment. Cells treated with MBO-asGCS were used to settle the parameters and all the cells are in the quadrant 1 (Q1). (f) Stained cells for MBO-asGCS treatment. Cells treated with MBO-asGCS were incubated with FITC-CD24 and Alexa647-CD44 antibodies. The BCSC population in the quadrant 2 (Q2) is $13.84 \pm 0.4\%$, from triplicate assay; $P < 0.001$ as compared to the vehicle (b).

Fig. 4.

Fluorescent immunostaining of CD44⁺/CD24^{-/low} cells. MCF-7/Dox cells were treated with MBO-asGCS (200 nM, 48 h) or vehicle (lipofectamine) and stained with BCSC markers. *Green* represents the Alexa488-CD24; *red*, Alexa555-CD44; *blue*, DAPI. *Arrow heads* indicate the CD44⁺/CD24⁻ cells of BCSCs.