Flow cytometry analysis of human islet cell expression of heparan sulfate (HS) and collagen type XVIII (Col18)

Sarah Popp,Charmaine Simeonovic

Abstract

Isolated human islets were dispersed into single cells using Accutase (Millipore), ~1500-2000 islet equivalents/ml. 20,000-65,000 islet cells were transferred to individual wells of a 96 well culture plate (CELLSTAR, Greiner Bio-one) for immediate staining for flow cytometry analysis or for culture prior to staining. For intracellular staining, isolated islet cells were fixed in 2% paraformaldehyde (Sigma-Aldrich) and permeabilized using 0.3% saponin (Sigma-Aldrich). The cells were stained with 10E4 mouse anti-human HS mAb (10E4, 1/50; US Biological/Amsbio), mouse anti-mouse Col18 mAb (1/50; Santa Cruz Biotechnol.) or the corresponding isotype control Ig (mouse IgM_{κ} or $IgG_{2b\kappa}$; BD Biosciences) followed by goat anti-mouse Ig-R-PE (1/100; Southern Biotech). The geometric mean fluorescence ratio (GMFR) was calculated by dividing the geometric mean fluorescence intensity (GMFI) of cells stained with primary mAb by the GMFI obtained with the relevant isotype control Ig. Cells were analyzed using a BD LSRI flow cytometer and CellQuest™ Pro software (version 6.0; BD Biosciences).

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Guidelines

10E4 anti-heparan sulfate (HS) mAb identifies highly sulfated HS localised in human beta cells but does not identify the less sulfated HS in alpha cells.

Reference:

Theodoraki A, Hu Y, Poopalasundaram S et al (2015) Mol Cell Endocrinol 399: 296-310.

Before start

Before starting:

Materials:

1. Prepare:

(i) 2% Paraformaldehyde:

Add 2 g Paraformaldehyde (#P6148 Sigma) to 50 ml deionised water, a stirrer and 2 drops of 10 M NaOH. Heat at 60°C for 30 mins. Add 40 ml deionised water and 10 ml of 10x PBS and pH to 7.2. Store solution at 4°C.

(ii) PBS/3 mM EDTA:

112 mg EDTA (AJAX #180) in 100 ml PBS, sterile filter using 0.2 µm disposable filter.

(iii) Beta cell culture medium:

RPMI 1640 (Sigma #R0883) 200 ml

Heat-inactivated fetal calf serum (HIFCS) 20 ml

L-Glutamine (Gibco #25030081 200 mM) 2 ml (final 2 mM)

Penicillin G (MP Biomedicals #02194537), 0.06 mg/ml

Streptomycin (Sigma #S9137), 0.10 mg/ml

Neomycin (Sigma #N6386), 0.10 mg/ml

(iv) PBS/5% HIFCS:

500 ml PBS + 25 ml HIFCS

(v) PBS/5% HIFCS/0.3% saponin:

100 ml PBS/5% HIFCS + 300 mg saponin (#S7900, Sigma) and sterile filter with a disposable 0.2µm filter.

(vi) PBS/5% HIFCS/0.03% saponin:

10 ml PBS/5% HIFCS/0.3% saponin + 90 ml PBS/5%HIFCS

2. Mabs and pAbs:

10E4 (anti-HS) mAb, Amsbio #370255-1

Mouse anti-mouse collagen type XVIII (Col18A1), Santa Cruz Biotechnol #1837-46

IgM_{κ} , BD Biosciences #550340

Mouse $IgG_{2b\kappa}$, BD Biosciences #557351

Goat anti-mouse Ig R-PE, Southern Biotech #1010-09

3. Other reagents/materials:

Accutase, Millipore #SCR005

Cell culture plates: Cellstar #650180 (Greiner Bio-one)

Cluster tubes, Fisher Biotech #MTS-11C

Protocol

Step 1.

See Guidelines, “Before starting” and “Safety Warnings”.

Step 2.

Centrifuge human islets at 300g for 2 min at 23°C. Pour off the supernatant. Resuspend in 25 ml PBS/3 mM EDTA. Centrifuge at 300g.

Step 3.

Resuspend the islets in PBS/3 mM EDTA and transfer islets to 15ml tubes, 2000 IEQ/tube. Centrifuge at 300g then carefully remove the supernatant.

Step 4.

Gently resuspend each pellet in 1ml pre-thawed Accutase and place tubes in 37°C waterbath for 10 mins (Note: at 4 min and 8 min, gently knock the pellet to resuspend the islets)

Step 5.

Dissociate the islets by pipetting up and down 10-15 times using a 1 ml single channel pipette.

Step 6.

Add 10 ml culture medium to each tube to terminate the Accutase reaction and centrifuge for 5 min at 300g.

Step 7.

Discard the supernatant, pool the cells into a single 15 ml tube and determine cell density (using hemocytometer). Adjust cell density to 100,000 - 325,000 cells/ml.

Step 8.

Transfer islet cells to culture plate, 20,000 - 65,000 cells in 200 µl/well.

Step 9.

Centrifuge cells at 300g for 3 min at 23°C. Remove supernatant by flicking.

Step 10.

For intracellular staining, resuspend separate wells of islet cells in 100 µl 2% Paraformaldehyde. Treat for 10 min at room temperature. Add 100 µl PBS/5% HIFCS and spin again at 300g for 3 min at 23°C.

Step 11.

Flick off the supernatant and wash the cells in 200 µl PBS/5% HIFCS and centrifuge at 300g for 3 min at 23ºC.

Step 12.

Incubate cells for 30 min on ice with:

(i) 25 µl/well of 10E4 anti-HS mAb or mouse IgM (isotype control) diluted to 20 µg/ml with PBS/5% HIFCS/0.3% saponin;

or

(ii) 25 µl/well of anti-Col18 mAb or mouse IgG_{2b} (isotype control) diluted to 4 µg/ml with PBS/5% HIFCS/0.3% saponin;

Protect from light.

Step 13.
Wash 2x with PBS/5% HIFCS/0.03% saponin, as for Step 9.

Step 14.

Incubate cells for 30 min on ice with 25 µl/well of goat anti-mouse Ig PE diluted to 5 µg/ml with PBS/5% HIFCS/0.3% saponin. Protect from light.

Step 15.
Wash 2x with PBS/5% FCS/0.03% saponin, as for Step 9.

Step 16.

Resuspend cells in 100 µl/well of PBS/5% HIFCS, transfer cells from each well to an individual cluster tube and run samples on flow cytometer.

Excitation/emission wavelength: R-PE 565 nm/575 nm

Step 17.

For cell surface staining on separate aliquots of cells, apply steps 8, 9, 12-16 (inclusive), **with the exception that all washes and antibody dilutions are done in PBS/5% FCS**.

Step 18.

Analyse cell surface or intracellular HS or Col18 staining using CellQuest™ Pro software (version 6.0; BD Biosciences).

Step 19.

NOTE: To determine % islet cells that are beta cells, apply steps 8 and 9. Resuspend separate well(s) of cells in 10 µM Newport Green, 100 µl/well. Incubate at 37°C for 1 hr. Add 100 µl PBS, centrifuge at 300g. Remove culture supernatant and resuspend in 100 µl PBS for flow cytometry analysis. Analyse flow cytometry data using CellQuest™ Pro software (version 6.0; BD Biosciences) to identify % of total islet cells that are Newport Green+ve beta cells.

Step 20.

Warnings

All handling of human islets is done in a Class II Biological Safety Cabinet.