Isolation of human islet cells, culture with heparan sulfate mimetics and flow cytometry analysis of beta cell viability

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Abstract

Isolated human islets were dispersed into single cells using Accutase (Millipore), ~1500-2000 islet equivalents (IEQ)/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. Isolated human islet cells were cultured in the presence or absence of the HS mimetics heparin (a highly sulfated HS analogue from porcine intestinal mucosa), BT548 (a glycol split low molecular weight heparin (LMWH; 3 kDa) lacking anti-coagulant activity) or PI-88 (Progen Pharmaceuticals Limited,) at 50 µg/ml for 2 days in 5% CO_2, 95% air at 37ºC. In some studies islet cells were acutely treated with 30% H_2O_2 (Chem-Supply) as a source of reactive oxygen species (ROS) for 5 min on day 0 or after culture for 2 days with/without HS mimetics. Beta cells were identified by staining with Newport Green (NG; 10 µmol/L; Invitrogen, Molecular Probes), a fluorescent probe that detects zinc in the insulin granules of beta cells. Damaged and dying islet cells were assessed using 7-Aminoactinomycin (7AAD, 10 µg/ml; Life Technologies) or by Sytox green (31.25 nmol/L; Invitrogen, Molecular Probes) uptake. Cells were analyzed using a BD LSRI flow cytometer and CellQuest™ Pro software (version 6.0; BD Biosciences).

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Before start

Materials:

1. Prepare:

(i) PBS/3mM EDTA:

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

(ii) 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

2. Other reagents:

Accutase, Millipore #SCR005

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

Newport Green DCF diacetate (Newport Green), Invitrogen, Molecular Probes #N7991

7-Aminoactinomycin (7AAD) Life Technologies #A1310

SYTOX Green, Invitrogen, Molecular Probes #S7020

Hydrogen peroxide (30% w/w), Chem-Supply Pty Ltd (Australia) #HA154-500M

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 islet equivalents (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. Centrifuge at 300g then remove the supernatant by flicking.

Step 9.

Islet cells are cultured with heparin or heparan sulfate mimetics (e.g. PI-88) at a final concentration of 50 µg/ml in 200 µl/well. Control cells are cultured in medium.

Step 10.

Cell viability is determined on day 0 and 2 days after culture by staining with Newport Green /7AAD or SYTOX Green followed by flow cytometry analysis:

(i) For Newport Green/7AAD staining, centrifuge culture plate at 300g for 3 min and remove culture supernatant. Resuspend cells in 10 µM Newport Green, 100 µl/well. Incubate at 37°C for 1 hr. Add 100 µl culture medium, centrifuge at 300g. Remove culture supernatant and resuspend in 10 µg/ml 7AAD, 100 µl/well. Incubate at 37°C for 15 min. 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). Viable beta cells are Newport Green+ve 7AAD-ve; dead/damaged beta cells are Newport Green+ve, 7AAD+ve.

Excitation/emission wavelengths:

Newport Green: 503 nm/535 nm

7AAD: 546 nm/647 nm

(ii) For monitoring hydrogen peroxide-induced cell death, centrifuge culture plate at 300g for 3 min and remove culture supernatant. Resuspend cells in 100 µl of 30% H_2O_2 or culture medium for 5 min. Add 100 µl culture medium and centrifuge at 300g for 3 min. Remove culture supernatant and resuspend cells in 31.25 nM SYTOX Green (1/160,000 dilution of stock), 100 µl/well. Incubate at 37°C for 15 min. Add 100 µl PBS, centrifuge at 300g for 3 min. Remove culture supernatant and resuspend cells in 100 µl PBS for flow cytometry analysis. Analyse flow cytometry data using CellQuest™ Pro software (version 6.0; BD Biosciences). Dead/damaged islet cells are SYTOX Green+ve, compared to unstained controls.

Excitation/emission wavelength for SYTOX Green: 504 nm/523 nm

Step 11.

Warnings

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