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Comprehensive Analysis of Human Pancreatic Islets Using Flow and Laser Scanning Cytometry

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Abstract

Assessing islet cellular composition and β cell viability using Flow Cytometry (FC) and Laser Scanning Cytometry (LSC) may aid in determining the transplant quality of islets.

Human islets (2500 IEQ, n = 44, purity ≥80%) dissociated into a single cell suspension were stained with ductal marker CA19, with Newport Green (NG) and FluoZin3 (FL3) for β-cell identification, with TMRE to assess mitochondrial membrane potential, with DAPI to identify live vs. dead cells, and with Annexin-V/DAPI to differentiate apoptotic and necrotic cells. For LSC, cell preparations (n = 9) were stained for insulin (β-cells), glucagon (α-cells), somatostatin (δ cells), and pancreatic polypeptide (ppp cells). Fluorescence microscopy (EtBr/FDA) and insulin response were also measured. DAPI− staining was 73.78% ± 1.37, while EtBr/FDA was 96% ± 0.48. 52.5% ± 3.73 of all cells were NG+, of which 58.08% ± 2.61 were NG+/TMRE+. Annexin-V/DAPI staining (n = 26) showed 13.8% ± 0.89 apoptotic, 27.2% ± 2.0 necrotic, and 51.9% ± 2.22 live cells. 26.0% ± 5.19 of cells were CA19 positive (n = 17), of which 45.5% ± 4.37 were also TMRE+, and 5.2% ± 1.2 of the TMRE+ were also NG+/CA19+. NG and FL3 showed similar staining (n = 8). Comparison of short-term (≤2 days) versus long-term (≥3 days) culture showed similar TMRE+/NG+ averages, albeit lower percentages of live (36.4% vs 51.9%), and higher percentages of apoptotic (19.2% vs 13.8%) and necrotic cells (37.4% vs 27.2%) for long-term, as determined by Annexin-V staining. LSC resulted in 54.17% ± 4.62 β-cells, 33.33% ± 4.16 α-cells, 8.75% ± 2.5 δ-cells, and 3.75% ± 0.79 ppp cells. There is no significant difference between insulin positive cells and NG positive cells ($P \leq .55$). FC and LSC provide valuable information about islet quality, which could potentially be used for evaluating islets prior to transplantation.

Clinical islet transplantation is an effective procedure to reverse diabetes in selected type 1 diabetic patients with uncontrollable glycemia,[1–5] as confirmed by multicenter trials.[3] However, only a few experienced centers have been able to duplicate the Edmonton protocol; possibly due to the lack of a reliable method to evaluate islet potency prior to transplantation. This observation indicates a critical need to develop proper potency screening to evaluate islet quality in order to avoid transplanting suboptimal islets. The focus

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of this study was to assess islet (β-cell) viability using Flow Cytometry (FC) and cellular composition using Laser Scanning Cytometry (LSC).

MATERIALS AND METHODS

Islet Cell Processing and Culture

Human islets were isolated from 44 cadaveric pancreata utilizing standard islet processing and Liberase HI (Roche Diagnostics, Roche Applied Science, Indianapolis, IN).[2–4,6,7] Dithizone (DTZ) staining was used to count the islets which was expressed as Islet Equivalents (IEQ). Islets cultured in serum free medium (pH 7.4) using T175 non-tissue treated flasks were incubated at 37°C/5% CO_2 for 18 to 24 hours and then at 30°C for up to 72 hours.

Dissociation and Staining

Aliquots of 2,500 IEQ with purities ≥ 80% were utilized for FC and LSC. Cells washed twice with PBS (0.1% HSA, 2 mmol/L $MgCl_2$) were suspended in prewarmed TrypLE (Gibco, Invitrogen Corporation, Carlsbad, CA) to dissociate the islets. Digestion was stopped with 1 mL cold fetal calf serum (FCS). Washed Cells were filtered twice to remove undigested tissue. Single cells were resuspended in PBS and divided into 10 μl aliquots for individual staining and LSC. Cells were stained using: Newport Green (NG)[6,7] or FluoZin3 (FL3, Invitrogen Corp, Carlsbad, Calif),[8,9] which are zinc binding dyes that detect insulin-producing β-cells (1 μmol/L); 50 nM tetramethylrhodamine ethyl ester perchlorate (TMRE) for mitochondrial membrane potential; 4,6-diamidino-2-phenylindole dihydrochloride (DAPI - 0.2 μg/mL); Annexin-V-FLUOS (Annexin-V 0.1 μg/μL, Indianapolis, Ind), or mouse monoclonal antibody Anti-CA19-9[10] (ductal cell marker) labeled with goat anti-mouse Alexa 647 (1:200 dilution). Cells were incubated in the dark using appropriate dye(s): Anti-CA19-9 and Alexa 647 (10 μL and 10 μg/mL) each for 15 minutes at 4°C, NG and TMRE for 60 minutes at 37°C, FL3, Annexin-V, and DAPI immediately prior to the FC run.

Flow Cytometry and Data Analysis

After staining, cells were immediately analyzed using the CyAn ADP Flow Cytometer (Dako). A minimum of 12,000 events were acquired. All events were analyzed using FlowJo software (version 5.7.1, or 6.2.1). A no stain control “auto” consisting only of cells in PBS was used to determine background. Single stain controls for NG, TMRE, DAPI, and CA19-Alexa 647 were utilized for compensation. Combinations used for simultaneous detection were: NG/TMRE/DAPI, FL3/TMRE/DAPI, Annexin-V/DAPI, and NG/TMRE/CA-19-Alexa 647/DAPI. Data were recorded as mean percentages ± standard errors of the mean (SEM).

Laser Scanning Cytometry

Immunofluorescent staining and LSC analysis were performed on 9 islet preparations. Dispersed single cells were resuspended in 100 μl of PBS. A 3–5 μL cell sample spread evenly onto a 10 mm circle on a glass slide was allowed to air dry at room temperature, and fixed for 15 minutes with 2.5% Paraformaldehyde diluted in PBS. Specimen slides were

then washed (2×) with buffer (BioGenex) and preserved at −20°C. On the day of staining, slides were allowed to equilibrate to room temperature, washed with buffer, and treated for 30 minutes with protein blocker to prevent non-specific binding. Double immunofluorescent staining used endocrine markers: mouse anti-insulin and anti-glucagon, rabbit anti-pancreatic polypeptide (ppp) and anti-somatostatin as primary (1°) antibodies. Secondary antibody (2°) labels were: goat anti-mouse Alexa®488 and goat anti-rabbit Alexa 647. Both 1° and 2° antibodies were incubated separately at room temperature for 20 minutes, washed thoroughly, and mounted using Prolong Gold Antifade (Invitrogen). The iCys Laser Scanning Cytometer (CompuCyte Corporation, Cambridge, Mass) and iCys Cytometric Analysis Software (version 3.2.1) with Watershed algorithm (to distinguish single events), were used for LSC analysis.

Viability and Functionality

Fluorescence microscopy, using Fluorescein Diacetate (FDA) and Ethidium Bromide (EtBr), was used to assess whole islet viability post-culture. In addition, insulin secretion (IS) was measured in 21 preparations. In brief, 200 IEQ were perifused for one hour at 37°C (equilibration), with buffer containing 1 mmol/L glucose, to establish baseline insulin release. Islets challenged with 16.7 mmol/L glucose and 25 mmol/L KCl had fractions collected at one minute intervals. Insulin was measured using an insulin ELISA Kit (Alpco Diagnostics, Salem, NH). Stimulation indices were calculated by dividing the average values of the initial responses over the average of the baseline.

RESULTS

Average viability of whole islets was 96% ± 0.48 using FDA/EtBr by microscopy, while dissociated single cell viability was 73.78% ± 1.37 using DAPI (FC). NG+ cells made up 52.5% ± 3.73 of all viable cells. Of these NG+ cells, 58.08% ± 2.61 were also TMRE+ cells, suggesting β cells with active mitochondria. Cells not staining for NG averaged 31.0% ± 2.73. On average 26% ± 5.2 of all cells stained as CA19+. Of these 45.5% ± 4.38 were also TMRE+, indicating active mitochondria. LSC resulted in 54.17% ± 4.62 insulin, 33.33% ± 4.16 glucagon, 8.75% ± 2.5 somatostatin, and 3.75% ± 0.79 ppp cells. Cells stained using NG/TMRE/CA19-Alexa/DAPI resulted in 4 different staining combinations: NG+/CA19− (35.1% ± 5.08), NG+/CA19+ (47.2% ± 5.31), NG−/CA19+ (12.4% ± 3.43), and NG+/CA19+ cells (5.2% ± 1.2). Apoptotic marker staining with Annexin-V/DAPI revealed 3 distinct populations of cells: 51.9% ± 2.22 live, 13.8% ± 0.89 apoptotic and 27.2% ± 2.03 necrotic cells. Annexin-V/DAPI results for short-term (ST-defined as cells in culture for 2 days or less) versus long-term culture (LT-cells in culture for 3 or more days) showed similar NG+/TMRE+ staining (ST 51.8% vs LT 51.7%) for both conditions but apoptotic testing resulted in more apoptotic and necrotic cells in the LT versus the ST culture (Live: ST 51.9% vs LT 36.4%, apoptotic: ST 13.8% vs LT 19.2%, necrotic ST 27.2% vs LT 37.4%). Table 1 shows these results. NG and FL3 showed similar staining results for some preparations but variability in others. The staining was more comparable when FL3 (0.5 μmol/L) was added just prior to the FC run (no incubation). Insulin stimulation results averaged 2.95 ± 0.2359.

DISCUSSION

Previous studies have shown that it is possible to assess islet cellular composition using single cells.[6–8] Staining of individual preparations was consistent, yet the viability of whole (96%) and dispersed (74% DAPI−) islets was significantly different, probably attributable to the FDA/EtBr method being a subjective microscopic procedure, compared to the automated FC procedure using single cells. Islets in early stages of apoptosis were indistinguishable with DAPI alone staining; however when using Annexin-V/DAPI, 13.8% of DAPI− cells stained as apoptotic (51.9% were live). Necrotic cells (27.2% ± 2.03), as determined by Annexin-V/DAPI, and dead cells (27.9 ± 1.87), as determined by DAPI stain alone (n = 26), yielded similar results. The results showed that NG and insulin staining were similar, confirming the previous findings that NG+ staining can be used for β-cell detection.[6,7] Staining with NG and CA19 yielded an interesting result: the presence of 5.2% NG+/CA19+ cells. Although long-term insulin independence still evades us, improved glycemic control and reduction of other complications associated with diabetes have been documented in islet transplant recipients.[3,4] Recent studies have reported that long term pancreatic function deteriorates post transplantation,[4,5] possibly due to several factors including autoimmune destruction of transplanted islets, toxicity of current immunosuppressive agents that prevent allograft rejection, or insufficient precursor cells which may have been removed prior to transplantation.[2–5] Therefore, assessing β-cell number, cellular composition, and apoptotic events in an islet preparation prior to transplantation may improve outcomes. FC and LSC in association with other assessment tools may aid in determining acceptable transplant quality of islet preparations.

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Table 1

Flow Cytometry Results for Dissociated Pancreatic Islets

N =	Stain Combinations	Cell Type	% of Total ± SEM
17	*NG/TMRE/CA19-Alexa647/DAPI*		
	CA19+	Ductal cells	26.0 ± 5.19
	CA19+/TMRE+	Ductal cells with MP*	45.5 ± 4.37
	TMRE+/CA19−/NG−	non-ductal, non beta cells with MP	35.1 ± 5.08
	TMRE+/CA19−/NG+	non-ductal, beta cell with MP	47.2 ± 5.31
	TMRE+/CA19+/NG−	ductal, non-beta cell with MP	12.4 ± 3.43
	TMRE+/CA19+/NG+	ductal & beta cell with MP	5.20 ± 1.20
44	*NG/TMRE/DAPI*		
	NG+	Beta cell characteristic	52.52 ± 3.73
	NG+/TMRE+	Beta cells with MP	58.08 ± 2.61
8	*FL3/TMRE/DAPI*		
	FL3+/TMRE+	Beta cells with MP	52.50 ± 5.56
44	*DAPI*		
	DAPI−	Live cell	73.78 ± 1.37
	DAPI+	Dead cell	26.08 ± 1.35
Short-term culture			
26	*Annexin/DAPI*		
	Annexin−/DAPI−	Live cells	51.90 ± 2.22
	Annexin+/DAPI−	Apoptotic cells	13.80 ± 0.89
	Annexin+/DAPI+	necrotic/dead cells	27.20 ± 2.02
26	*NG/TMRE/DAPI*		
	DAPI−	Live Cells	72.09 ± 1.87
	NG+/TMRE+	Beta cells with MP	51.80 ± 3.43
Long-term culture			
5	*Annexin/DAPI*		
	Annexin−/DAPI−	Live cells	36.40 ± 6.22
	Annexin+/DAPI−	Apoptotic cells	19.20 ± 5.27
	Annexin+/DAPI+	necrotic/dead cells	37.40 ± 1.69
6	*NG/TMRE/DAPI*		
	DAPI−	Live Cells	67.00 ± 0.93
	NG+/TMRE+	Beta cells with MP	51.70 ± 12.47

*MP = Membrane Potential.