A Novel Method for the Assessment of Cellular Composition and Beta-Cell Viability in Human Islet Preparations

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Introduction

Recent improvements in islet isolation and immunosuppression have made transplantation of human islets a viable treatment for patients with type 1 diabetes mellitus (1,2). While selected centers have reported high rates of success (1–5), there have been reports of failures occurring in the very early post-transplant period. This disappointing observation could be related to the use of islet preparations of less than optimal quality (6–8). This problem is linked to the lack of reliable markers of islet potency to screen human islet preparations prior to transplantation.

Currently accepted product release criteria include viability measured by DNA-binding dye exclusion (9), islet cell purity based on dithizone (DTZ) staining (10,11), and in vitro glucose-stimulated insulin release (11,12). According to these pre-transplant criteria, islet preparations that failed to reverse diabetes were indistinguishable from those that resulted in excellent function. DNA-binding dye exclusion can only reveal cells that have lost membrane perm selectivity. Additionally, this method does not identify apoptotic cells or determines whether dead cells preferentially belong to any given subset. Dithizone staining can only provide an estimate of endocrine cell content in islet preparations, but does not allow for the definition of β-cell content (11). A more predictive pre-transplant test is diabetes reversal in immunodeficient mice (11–13); however, several days are required for outcome assessment, making it an unpractical pre-transplant product release criteria.

There is, therefore, a critical need for alternative analytical methods to define islet cellular composition and fractional viability (including early events of apoptosis) before clinical transplantation. The Food and Drug Administration’s Biological Response Modifiers Committee has recently underlined the need for developing such methods (14,15).

We describe a novel method of analysis to precisely and objectively quantify cellular composition and fractional β-cell viability in human islets, based on the use of Laser Scanning Cytometry (LSC) and cytofluorimetry. Analysis of human islet preparations with these techniques allows...
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for the definition of β-cell mass and viability, and could be of assistance for potency testing of human islets before transplantation.

Research Design and Methods

Pancreas preservation and human islet isolation

Human islet isolations were performed at the Human Cell Processing Facility of the University of Miami School of Medicine from human pancreata preserved with either preoxygenated (30 min) two-layer perfluorocarbon/University of Wisconsin solution (PFC/UW) or with UW alone (16). Islets were isolated using a modification of the automated method (1,12,17). A total of 62 consecutive islet preparations were analyzed using the methods described below.

Cell dissociation

Single cell suspensions were obtained from human islets by incubating aliquots of approximately 1000–1500 islet equivalents (IEQ) (11,12) in 1 mL accutase solution (Innovative Cell Technologies, Inc, San Diego, CA) at 37°C for 10–15 min, followed by gentle pipetting. This method was selected after comparison with other techniques (trypsin-based and nonenzymatic buffers), as it provided consistent cellular dispersion, high yield and transducible viability loss (not shown).

Analysis of cellular composition by immunofluorescence

Dispersed cells were fixed on glass slides with 2.5% paraformaldehyde (Electron Microscopy Sciences, Washington, PA). After permeabilization with 1% saponin for 15 min, cells were incubated with Protein Block (BioGenex, San Ramon, CA) for 30 min, to reduce nonspecific binding. After washing in Optimax Wash Buffer (BioGenex, San Ramon, CA), cells were incubated for 1 h with the following antibodies: mouse monoclonal antibody to insulin (1:100), rabbit polyclonal antibody to somatostatin (1:100), from Neo Markers (Fremont, CA); mouse monoclonal antibody to glucagon (1:500; Sigma, St. Louis, MO); undiluted rabbit polyclonal antibody to pancreatic polypeptide (PP; Bio-Genex, San Ramon, CA). After washing, samples were incubated with either goat antimouse (Alexa Fluor 488 goat antimouse IgG, 1:200 dilution) or goat antirabbit (Alexa Fluor 488 goat antirabbit IgG, 1:200 dilution) antibodies, both from Molecular Probes (Eugene, OR). Omission of the primary antibody served as negative control. After washing, 4′,6-diamidino-2-phenyindole (DAPI) was applied to stain cell nuclei. Samples were analyzed using a LSC (CompuCyte, Cambridge, MA).

LSC analysis and data display

Data acquisition and analysis were performed using LSC at the Imaging Core of the Diabetes Research Institute. The LSC allows for fluorescence-based quantitative measurements on tissue sections or other cellular preparations at single-cell level. An optics/electronics unit coupled to an argon UV laser and DAPI detector. Each hormone-positive event was recorded using the argon laser and green (Alexa-488) detector. The following data were acquired: area, x position, y position, fluorescence integral and maximal intensity for all channels. Single cells were identified and gated according to the DAPI staining area (Figure 1B). Fluorescence intensity was recorded on a histogram (Figure 1C). Cells from every subpopulation were visualized directly in the LSC by relocation to confirm regular morphology. A minimum of 10 000 cells were acquired and analyzed for each sample.

Analysis of cellular composition on whole islet sections

Isolated islets were formalin fixed and paraffin embedded. Sections were cut and stained with hormone-specific antibodies (insulin, glucagon and somatostatin). Nuclei were counterstained with DAPI. Sections were analyzed with a confocal microscope (Zeiss LMS 520). Five independent human islet preparations were analyzed and compared to the cellular composition of dissociated islet cells in the same preparations assessed by LSC. At least five randomly selected fields per preparation were assessed. Beta-, α- and δ-cells were counted and percentages were calculated by using the DAPI staining to number all islet cells.

Assessment of β-cell content in dissociated islets

Beta-cell content within islets was calculated based on the analysis of immunostaining for endocrine markers by LSC using the formula:

\[
\frac{\beta-\text{cell\%}}{\beta + \alpha + \delta + \text{PP - cells} \times 100.}
\]

Determination of fractional β-cell viability

For assessment of apoptosis, single cell suspensions were incubated with 1 μM Newport Green PDX acetoxyxmethylene (NG; Molecular Probes) and 100 ng/mL of tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) for 30 min at 37°C in PBS without Ca2+ and Mg2+. Newport Green allows for the definition of cell subsets according to zinc content (18). TMRE selectively binds to mitochondrial membranes, allowing for detection of apoptosis (that results in decreased staining) (19,20). After washing, cells were stained with 7-aminoactinomycin D (7-AAD; Molecular Probes), which selectively binds to mitochondrial membranes, allowing for detection of cell death. Cell suspensions were analyzed (minimum 3.0 × 104 events) using a FACScan cytometer (Becton Dickinson, Mountain View, CA) with the CellQuest software.

Human β-cell sorting

Dispersed cells were stained with NG and 7-AAD. Cell sorting was performed using a FACSVantage (Becton Dickinson). Cell subsets 7-AAD+, NGbright and NGdimnegative were collected separately (18). Control analysis showed that FACS sorting led to >90% purity.

Delivery of pro-apoptotic stimuli to islet cells

Islets were treated with selected compounds to induce apoptosis. The nitric oxide (NO) donor sodium nitroprusside (Baxter Healthcare Corporation, Deerfield, IL) was used at 0.5 mM for 18 h. Hydrogen peroxide (H2O2; Sigma) was used at 200 μM for 18 h. IL-1β (50 U/mL) alone or in combination with TNF-α (1000 U/mL) and IFN-γ (1000 U/mL) was used for 24 h. In additional experiments β-cell stress was induced by incubation of islet aliquots maintained as a pellet in a 15 mL conical tube for either 6 or 18 h.

In vivo assessment of islet potency in the diabetic nude mouse model

Animal procedures were approved by the IACUC, and performed in the Preclinical Cell Processing Core. Male athymic nu/nu (nude) mice (Harlan Laboratories, Indianapolis, IN) were housed in virus-antibody-free rooms in microisolated cages, having free access to autoclaved chow and water. Animals were rendered diabetic via IV administration of 200 mg/kg of Streptozotocin (Sigma). Nonfasting blood glucose was assessed with a glucometer (Elite, Bayer; Tarrytown, NY). Mice with sustained hyperglycemia (>300 mg/dL) were used as islet graft recipients.
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Figure 1: Analysis of cellular composition in human islets by LSC. An aliquot of approximately 100 IEQ was dissociated to obtain single cell suspension (see methods). Single cells were divided into four individual aliquots, and each aliquot was stained with one of the indicated antibodies. The appropriate fluorochrome-conjugated secondary antibody was subsequently added. A. The desired area to be scanned was visually located using the microscope connected to the instrument, and the examined area was mapped using the Wincyte software. B. For removal of aggregated cells from further analysis, single cells were identified based on DAPI fluorescence emission area. C. Analysis with LCS allowed for the computation of the percentage of positive cells (cytoplasm stained in green, nucleus in blue by DAPI) in each preparation, by recording positive and negative cells (the latter revealed by blue nuclear staining in the absence of green cytoplasmic staining). The instrument software graphically depicts the results in the form of the plots shown here, where both percentage of positive events and events’ intensity are shown. The data shown is representative of more than 60 human islet preparations.

2000 human IEQ per recipient were transplanted under the kidney capsule (11,21,22) and nonfasting blood glucose values were assessed three times a week. Reversal of diabetes was defined as stable nonfasting blood glucose <200mg/dL. Nephrectomy of the graft-bearing kidney was performed to confirm return to hyperglycemia and exclude residual function of the native pancreas in animals achieving normoglycemia after transplantation.

Statistical analysis
Data were analyzed using Excel for Windows software for descriptive statistics and data plotting. Data are shown as mean ± standard deviation (SD). Statistical significance was considered for p < 0.05. Chi-square analysis was used to assess significant differences in the product of β-cell content (%) and viability (%) versus transplantation success in immunodeficient mice. Logistic regression was then used to explore the influence of the two factors separately.

Results

LSC allows precise definition of islet cell composition
As shown in Figure 1C, meaningful data can be acquired by the use of marker-specific immunofluorescence analysis. Figure 2A shows the correlation of β-cell percentages (LSC analysis) versus purity, the latter assessed by DTZ staining in 62 human islet preparations. The proportion of β-cells was much lower than expected based on DTZ staining, and quite widely disparate between preparations, even when ≥90% purity by DTZ staining was documented. We then calculated β-cell content in islets using the formula [(β-cell%) × (β + α + δ + PP-cell%)]⁻¹ × 100. The mean ±SD of β, α, δ and PP-cells was 51.3 ± 10.4, 36.0 ± 9.4, 8.0 ± 2.6 and 4.6 ± 4.7, respectively (Figure 2B). When analysis of cellular composition was performed on preparations obtained from pancreata preserved in UW, and compared to preparations from pancreata preserved in PFC/UW, no significant differences were observed (data not shown). These results indicate that analysis of islet purity by DTZ staining is largely inadequate, and that assessment of cellular composition by LSC represents a significant improvement toward objective evaluation.

An important question was whether LSC analysis of endocrine cell subsets alone would allow for discriminating between live and dead cells. Experiments were performed where β-cell stress (resulting in death of a large
percentage of cells) was induced by hypoxia/starvation. Islet cells maintained in pellet for 18 h have little oxygen and nutrients available to support their viability and function. After the incubation period, dead cells were identified by 7-AAD staining. Figure 3 shows that both live cells (control sample) and a sample containing mostly dead cells (78\% 7-AAD\(^+\)) were indistinguishable when stained with antiinsulin and antiglucagon antibodies, indicating that analysis with LSC, while providing invaluable information on cellular composition, was not sufficient to estimate \(\beta\)-cell viability.

**Newport Green staining allows identification of \(\beta\)-cells**

We next utilized the zinc-binding fluorochrome NG to identify \(\beta\)-cells in virtue of their high zinc content. Single islet cell suspensions were incubated with both 7-AAD and NG and then analyzed by FACS. After exclusion of 7-AAD\(^+\) cells from further analysis, a bimodal pattern of NG staining was observed, with \textit{bright} and \textit{dim/negative} cells, as shown in Figure 4A, upper left panel. Cells were then sorted into NG\textit{bright} and NG\textit{dim/negative} subsets (middle panels), and each subset was individually analyzed by immunofluorescence. Newport Green\textit{bright} cells appeared highly enriched for \(\beta\)-cells, while NG\textit{dim/negative} cells were highly depleted of \(\beta\)-cells (Figure 4A, right panels), comprising other endocrine subsets and nonendocrine cells. Beta-cell identification was confirmed by functional analysis of \textit{in vitro} glucose-stimulated insulin secretion: NG\textit{bright} cells responded, while NG\textit{dim/negative} cells had marginal insulin secretion (not shown).

The exclusion of 7-AAD\(^+\) (dead cells) from further analysis of NG staining could introduce a bias, excluding dead cells preferentially present in selected subsets. We therefore compared \(\beta\)-cell content of individual human islet preparations ascertained via FACS with that of LSC analysis. While the former excludes dead cells, the latter does not. There was a tight relationship between the results obtained with both techniques in all preparations tested (Figure 4B), indicating that dead cells did not preferentially belong to any particular subset. Furthermore, our data on
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Table 1: Cellular composition in whole islets and dispersed islet cells

<table>
<thead>
<tr>
<th></th>
<th>β-cells</th>
<th>α-cells</th>
<th>δ-cells</th>
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<tbody>
<tr>
<td>Nondissociated islet</td>
<td>57.4 ± 19.6%</td>
<td>32.8 ± 16.3%</td>
<td>9.8 ± 2.9%</td>
</tr>
<tr>
<td>Dissociated islet cells</td>
<td>54.4 ± 11.4%</td>
<td>34.7 ± 12.5%</td>
<td>10.9 ± 4.6%</td>
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Relative percentages of β, α and δ-cells in islets and dissociated islet cells obtained from five independent human preparations are compared. No statistically significant differences are observed.

set of experiments, β, α and δ-cell percentages of islets from five randomly selected preparations were very similar to those obtained with LSC on dissociated cells from the same preparations (Table 1).

TMRE staining of NG-bright cells allows definition of viable and apoptotic β-cells

We evaluated apoptosis selectively in the NG$^{bright}$ subset according to the method summarized in Figure 5. After exclusion of dead cells from analysis by 7-AAD staining, live β-cells (NG$^{bright}$) were analyzed for mitochondrial membrane potential by staining with TMRE. In a complementary fashion, NG$^{dim/negative}$ cells were also analyzed. This approach allowed for the definition of subsets of β-cells that differ in TMRE staining, discriminating between healthy and apoptotic β-cells (Figure 6). Control islets were compared to aliquots incubated in hypoxic conditions for 6 or 18 h. As expected, the proportion of TMRE+ β-cells increased as a function of time in hypoxic culture. However, the decreased TMRE staining in β-cells cultured in hypoxic condition for 6 h was not paralleled by a change in the percentage of 7-AAD+ cells. This difference could be of critical importance to evaluate viability and potency of islet preparations. This is a further suggestion that conventional methods of islet cell viability assessment based on cell membrane integrity may be insufficient.

A clear correlation in fact was observed between TMRE staining and reversal of diabetes in transplanted immunodeficient rodents (Figure 6) in preparations that were indistinguishable in terms of 7-AAD staining, indicating that analysis of viability with conventional methods could not always predict in vivo function; diabetes could not be reversed using islets cultured for 6 h in hypoxic culture, where 7-AAD viability indicated a percentage of dead cells comparable to controls (29% vs. 27%, respectively). The additional analytical step with TMRE was necessary to predict functional outcome in the 6-h pellet group (Figure 6). A predictive value of DNA-binding dyes (7-AAD) could easily be observed in extreme hypoxic conditions (18-h pellet).

Analysis of β-cell viability/apoptosis reveals the noxious effects of pro-inflammatory stimuli

While the use of hypoxia/starvation is a quick and effective method to decrease islet cell viability and induce apoptosis,
we wanted to confirm the value of our islet assessment method when other noxious condition were utilized. We evaluated the effects of reactive oxygen species (ROS), nitric oxide (NO) and cytokines, using the same method for analysis of β-cell apoptosis. All the selected noxious conditions utilized (SNP, H$_2$O$_2$, IL-1β and the cocktails of IL-1β, TNF-α and IFN-γ), led to an increase in the percentage of apoptotic β-cells (Figure 7A), suggesting that our analytical method might be widely applicable.

**Beta cell-specific analysis of viability/apoptosis allows precise quantification regardless of sample purity**

Islet aliquots with different degrees of purity from 62 preparations were assessed with our method. In the representative sample of more than 100 independent experiments (Figure 8A), TMRE$^+$ cells in total living cells (7-AAD$^-$ population) of preparations A, B and C were 61%, 67% and 50%, respectively (Figure 8A, top 3 panels). These results were subsequently compared with β-cell-specific viability (Figure 8A, bottom panels), where percentages of TMRE$^+$ β-cells were 80%, 49% and 82%, respectively. This remarkable discrepancy clearly shows the advantage of selectively analyzing β-cell viability by combining NG and TMRE staining. Analysis of NG$^{bright}$ percentage (Figure 8A, center panels) allows for the definition of β-cell mass in the preparation, and analysis of NG$^{bright}$ TMRE$^+$ percentages allows for the definition of the viable β-cell mass (Figure 8A, bottom panels). Both parameters contribute significantly to the predictive value of our test. In more than 100 samples obtained from 62 human islet preparations with different purity, β-cells viability did not always correlate to other cells’ viability and β-cell content.

Furthermore, aliquots of 2000 IEQ obtained from 24 individual islet preparations were transplanted to 82 diabetic immunodeficient mice. Beta-cell content (%) and β-cell fractional viability (%) were measured and plotted for each preparation, and related to transplant success or failure (Figure 8A). This data analysis showed that success rate was higher when preparations had higher β-cell content and higher viability. The two values [β-cell content (%) and β-cell fractional viability (%)] were used to obtain a numeric product (β-cell viability index). This was then analyzed to seek a relationship with the in vivo assessment of islet potency (Figure 9B). We show here that transplantation success rate has an evident relation with β-cell viability index. Chi-square analysis of β-cell viability index categories (<0.2, between 0.2 and 0.3, between 0.3 and 0.4 and >0.4) versus success overwhelmingly suggests a positive association between index and success rate (p < 0.0001); Table 2 details the number of transplants and the success rates in the selected index categories. Logistic regression was then used to explore the two factors separately. When adjusting for β-cell content (%) in islets, β-cell fractional viability is an independent predictor that is significantly positively associated with success rate. The odds of success corresponding to a 10% increase in fractional viability is
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Figure 6: Comparative analysis of cell viability, β-cell apoptosis and in vivo islet function. Islet aliquots were either cultured in conventional conditions (upper panels), or cultured in conditions leading to hypoxia/starvation (pellet) for 6 h (middle panels) or 18 h (lower panels). Analysis of cell viability by conventional means was performed on whole cells by 7-AAD staining (left panels). Analysis of β-cell apoptosis was performed by analysis of TMRE staining on NG^bright^ cells (vertical middle panels). Analysis of in vivo function was performed by transplantation in diabetic immunodeficient rodents (right panels). The figure shows that a 6-h culture in hypoxia/starvation resulted in an increase in the percentage of apoptotic β-cells (reduced % of TMRE^+^, NG^bright^ cells), and in loss of function in vivo, while analysis of cell viability by conventional means showed no difference, when compared to the control. When 18-h hypoxia/starvation was used, also nonselective analysis of cell viability reveals the detrimental effect of such treatment. This data argues in favor of a higher sensitivity of our novel analytical method as a predictive test of in vivo function, when compared to nonspecific viability assays based on DNA-binding dye exclusion. Results shown are representative of at least 5 independent experiments, where three mice per condition were transplanted.

3.78 (2.01, 7.08, p < 0.0001) times greater for a fixed fraction of β-cells.

Discussion

In the present study, we demonstrated that a novel analytical method based on LSC and FACS analysis of islet preparations resulted in significant advantages, compared to the currently used techniques, providing objective information on islet cellular composition and islet cell subset viability (in particular β-cells).

LSC analysis of cellular composition appears as the most objective and sensitive means to determine the relative percentage of different cell subsets in islet preparations. More conventional techniques such as immunohistochemical (23,24) and electron microscopic (EM) analysis rely on subjective interpretation of the results and scoring based on observation of samples by the operator(s). The LSC hardware and software provide objective analysis of substantially higher number of cells (e.g. >10 000 cells) in a short time, as compared to the few hundreds that are generally counted manually or by operator-assisted imaging analysis.

Our results underline the inadequacy of DTZ staining to estimate β-cell mass, as preparations with similar purity assessed by DTZ actually contained significantly different β-cell masses (up to two-fold). While β-cell mass estimates are clearly a step forward in the definition of a dose–response relationship with transplantation outcome, the data presented here suggests that analysis of β-cell viability by TMRE is of critical value for prediction of transplant outcome. Our results clearly indicate that there is a correlation between viable β-cell mass and transplantation outcome in immunodeficient rodents, which is considered to date the most reliable in vivo biologic test to assess potency of an islet preparation. Our finding that DNA-binding dye exclusion does not always correlate with graft outcome is another strong argument to suggest the inadequacy of this test, when performed as the only assay of islet viability.

We also show that our method to evaluate β-cell viability is capable of detecting damage mediated by different noxious conditions, including ischemia/hypoxia, H_2O_2, NO, IL-1β and cytokine cocktails (25–27). This suggests that the method is sensitive enough to be of assistance in the detection of islet cell damage possibly resulting from different conditions related to donor brain death, pancreas procurement and preservation, as well as islet processing.
Figure 7: Analysis of β-cell apoptosis after delivery of noxious stimuli. Islets were incubated in the presence or absence of the indicated noxious stimuli: sodium nitroprusside (SNP), an NO donor (A); H₂O₂ (B); IL1-β (C) and IL1-β, TNF-α and IFN-γ (D). Apoptosis was analyzed by TMRE staining in the beta cell (NGbright) subset. Incubation of islet cells in any of the four conditions resulted in increased apoptosis in β-cells, suggesting a selective pro-apoptotic effect of the studied compounds on insulin-producing β-cells. Additionally, these results show that our method of islet β-cells viability could be widely applicable also in experimental conditions that arguably mimic events occurring in vivo at the transplant site. Data is representative of at least five independent experiments in each experimental condition.

Figure 8: β-cell-specific analysis of viability/apoptosis in islet preparations with different purity. Islet aliquots with different degrees of purity from more than 60 preparations were assessed with our method. Representative data comparing three preparations (I, II and III) with different degrees of purity is shown. After gating the 7AAD⁺ cell population, percentages of TMRE⁺ cells in total living cells (top panels), of NGbright cells (β-cells; middle panels), and of TMRE⁺ cells within the NGbright cells (β-cells fractional viability; bottom panels) were analyzed. These data suggests that assessment of overall viability in islet cells may not represent an adequate estimate of β-cell viability: islet preparation II would have led to overestimation of β-cells viability, while islet preparation III would have led to underestimation. Data is representative of more than 100 independent experiments.
Figure 9: Predictive value of β-cell content/viability on in vivo islet function. Aliquots of 2000 IEQ from 24 individual islet preparations were transplanted to each of 82 diabetic immunodeficient mice. Figure 9A shows a plot of all transplanted preparations where β-cell content (%) and β-cell fractional viability (%) are recorded and related to transplant success. There is a clear trend to increased success rate when β-cell content and fractional viability are higher. In figure 9B, the product of β-cell content (%) and β-cell fractional viability (%) was calculated (β-cell viability index) and related to in vivo assessment of islet potency. It is clear that the higher the β-cell viability index, the better the transplant outcome. Statistical analysis also suggests that β-cell fractional viability is an independent predictor that is significantly positively associated with success rate (please see text).

Table 2: Relationship between beta cell viability index and in vivo transplantation success rate (reversal rate)

<table>
<thead>
<tr>
<th>Index</th>
<th>Number</th>
<th>Reversal rate</th>
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<tbody>
<tr>
<td>X ≤ 0.2</td>
<td>13</td>
<td>0%</td>
</tr>
<tr>
<td>0.2 &lt; X ≤ 0.3</td>
<td>30</td>
<td>30%</td>
</tr>
<tr>
<td>0.3 &lt; X ≤ 0.4</td>
<td>16</td>
<td>69%</td>
</tr>
<tr>
<td>0.4 &lt; X</td>
<td>23</td>
<td>100%</td>
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X = β-cell content in islet × β-cell viability.

Our choice of the reagents used in the FACS three color analysis was based on their full compatibility in terms of wave length emission, so that each can be easily discriminated, while providing the needed information. Furthermore, the selection of NG was based on recently reported high specificity and sensitivity for β-cells (18), and extensive literature review indicated that TMRE could be a suitable marker for the study of mitochondrial membrane potential (19,20). Our data argues in favor of the efficacy of this novel method in selectively detecting β-cell viability, and suggests that a correlation exists between β-cell fractional viability and in vivo function.

Additional methods to predict islet function have been proposed, including analysis of oxygen consumption (28) and analysis of ATP levels (29,30). While the theoretical premises to utilize these methods are certainly strong, we feel that there could be difficulties in using them as single, straight-forward analytical tests of islet potency. The major problem that characterizes both tests is that the read-outs do not allow for the definition of β-cell contribution to the overall result, since all other endocrine cells within the islets and contaminant nonendocrine cells present in the preparation contribute to oxygen consumption and ATP levels. For example, in islets with a purity of 50% (50% nonendocrine tissue) and a β-cell composition of 35% (35% of endocrine cells are β-cells), the contribution of β-cells to oxygen consumption or ATP levels could be approximately 18% of the total. If β-cells are 100% or 30% viable, the relative change in oxygen consumption could be approximately 13% of the total. Our data also suggests that assessment of the overall viability in islet cells poorly correlates with β-cells viability, and rather leads to either under- or overestimation of β-cells fractional viability. Nevertheless, integration of cellular composition assessment to discriminate the contributions of β- and non-β-cells with methods able to determine oxygen consumption or ATP contents will represent a valuable strategy to improve our analytical analysis in the near future.

Our method is based on the analysis of single cell suspensions obtained by dissociation of human islets with mild enzymatic treatment. It could be argued that the methodology used to disperse islets into single cells could introduce artifacts, such as the selective damage/loss of β-cells, therefore somehow lessening the value of our analysis. The proportion of β-cells in islets assessed in our study averages approximately 52%, which is consistent with what reported by other groups utilizing similar immunostaining techniques to characterize cellular composition of human islet preparations (31,32). In addition, our data on cellular composition are in keeping with data obtained with morphometric analysis of sections of isolated human islets where endocrine cell subsets were evaluated after immunofluorescence staining by confocal...
Imaging. In this set of experiments, β, α, and δ cell percentages were very similar to those obtained with LSC on the same preparations after dissociation. While alternative analytical approaches have been proposed to assess islet cell composition in whole islets such as EM, we would like to argue that both methods can be of value in the analysis of islet cell composition, and that the method that we describe here could indeed be a valid predictive test of islet functional performance, since we show that there is a correlation between β-cell viability and in vivo function.

If indeed our dispersion procedure selectively damages β-cells, then it could be argued that our analytical criteria are making evaluation of a preparation more stringent that it might need to be, but it would certainly not introduce the risk of utilizing less than optimal preparations for clinical use.

Advantages of our method include the fact that we can determine β-cell mass and viability in a very short time, in a fully objective manner, on large size samples (>30 000 cells), with little expense, and by the use of an instrument (flow cytometer) that is readily available to most facilities that isolate islets for research and transplantation.

In summary, we report a novel method for the assessment of cellular composition and β-cell viability in human islet preparations. This method for islet assessment may be of assistance for the identification of pre-transplant product release criteria that are predictive of post-transplant outcome, as recommended by the Food and Drug Administration before a biologic license for islet cell products could come, as recommended by the Food and Drug Administration.

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