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The Use of the BD Oxygen Biosensor System to Assess Isolated Human Islets of Langerhans: Oxygen Consumption as a Potential Measure of Islet Potency

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The measurement of cellular oxygen consumption rate (OCR) is a potential tool for the assessment of metabolic potency of isolated islets of Langerhans prior to clinical transplantation. We used a commercially available 96-well plate fluoroprobe, the BD Oxygen Biosensor System (OBS), to estimate OCR in 27 human islet preparations, and compared these results to those of concurrent mouse transplantations. OCR was estimated both from the dO_2 at steady state and from the transient rate of change of dO_2 during the initial culture period immediately after seeding ("dO_2 slope"). To demonstrate the validity of the OBS-derived values, it was shown that they scaled linearly with islet equivalent number/DNA concentration and with each other. These measurements were obtained for each preparation of islets incubated in media supplemented with either low (2.2 mM) or high (22 mM) glucose. Concurrently, one to three athymic nude mice were transplanted with 2,000 IEQs under the kidney capsule. The OCR Index, defined as the ratio of the DNA-normalized "dO_2 slope" in high glucose to that in low glucose, proved highly predictive of mouse transplant results. Of the 69 mice transplanted, those receiving islets where the OCR Index exceeded 1.27 were 90% likely to reverse within 3 days, whereas those receiving islets with an OCR Index below 1.27 took significantly longer, often failing to reverse at all over a 35-day time period. These results suggest that the OBS could be a useful tool for the pretransplant assessment of islet cell potency.

Key words: Oxygen consumption rate; Islets; Islet potency; Transplantation

INTRODUCTION

The recent clinical outcomes of islet cell transplantation highlight the need for a reproducible potency test for isolated islets, to ensure that optimal cellular products are utilized for clinical procedures (5,18,24,26–28). The accepted "gold standard" for assessing human islet function, concomitant renal subcapsular transplant in athymic nude mice, is not ideal because the results are only available after the cells have been transplanted into the human recipient, and the data are often difficult to extract regarding islet potency given the variations in IEQ number transplanted, the number of donors used for transplantation, and in the recipient characteristics. However, there has been some correlation demonstrated when single donors are utilized (10). However, it is accepted by most in the field that if the islets are functional at reversing hyperglycemia in the mouse model, they will function with a degree of similarity in human recipients, barring immunological and other issues. Clinicians need a quick and simple in vitro assay that is predictive of transplant success and that can be performed before the surgery (http://www.idsoc.org/committees/transplant/2004Transplant.html).

Historically, insulin release and viability staining have been utilized as a benchmark of islet function and well-being, though recent evidence suggests viability staining is a poor predictor of clinical outcome (2,19). Elaborate fluorescent/luminescent probes and sorting instruments have also been used to determine islet viability through membrane integrity/apoptosis staining, ATP quantification, and cell subset analysis. However, no

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single measurement has emerged as a reliable indicator of islet transplant outcome (2,13).

One in vitro assay more recently proposed for the assessment of islets is the measurement of oxygen consumption rate (OCR) (1,4,8,15,20,21,29–34). Relative to traditional membrane permeability staining methods for assaying cell viability, OCR could potentially be far more predictive of future functional viability, because changes in metabolic function precede actual changes in viability (19).

Historical methods for measuring OCR require cumbersome laboratory equipment, dive galvonometers, and microelectrodes, which preclude routine clinical use. The development of reliable fluorescent and phosphorescent fiber optic oxygen sensors has led to greater sensitivity and accuracy, and may enable user-friendly measurements of OCR to be collected in a clinical setting.

The most common current method for assessing OCR involves the use of fluorescent O_2 sensors and well-stirred submilliliter tanks, like those of Instech, Inc. (Plymouth Meeting, PA) (21). While this setup affords a direct and highly accurate measurement of OCR, it can be cumbersome in practical use. By contrast, the BD OBS provides an easily adapted, reproducible platform for measuring estimates of OCR, requiring only a conventional microplate fluorometer to read oxygen-dependent fluorescent signals. Moreover, the 96-well plate format allows for the concurrent testing of a variety of culture conditions in replicate.

The intent of the current study was to develop a rapid method for determining potency of islet cells. In doing so, it was necessary to recognize the potential pitfalls of the open system, foremost the lengthy transient state prior to achievement of diffusional steady state and the possibility of underestimating steady-state consumption values due to diffusional limitations. The validity of the OBS-derived measurements was demonstrated empirically via the linearity between both and cell number/DNA concentration.

The current work considers the ratio of OCR in high- versus low-glucose media. These ratios are derived from the slope of the transient change of oxygen concentration in the early period immediately after cell seeding. The use of such stimulation indices has long been demonstrated as a mark of islet cell viability and function (16,25). While some debate persists about the precise significance of such glucose responsiveness and the impact of media composition on it, it has been repeatedly shown to correlate to other metrics of islet potency (9,32).

The first objective of the current study was to determine whether the OBS was able to yield meaningful numbers representative of true OCR. This was accomplished via confirmation that each metric utilized—the early transient slope measurements and steady-state equilibrium measurements—varied empirically/linearly with cell number/DNA concentration under conditions where oxygen diffusion was not limiting. As such, dimensionless ratios of OCR can be taken. The second objective was then to determine whether or not OCR Index values were predictive of the length of time to achievement of normoglycemia in transplanted mice. This empirically linear variation of OCR metrics with time and with cell number has been demonstrated using the OBS in prior publications (3,4,8,33). Absent any diffusional limitation it can be theoretically assumed that true OCR also scales with cell (IEQ) number or DNA concentration (14). Because both metrics obtained from the OBS system (dO_2 slope, equilibrium OCR) scale with cell (IEQ) number and DNA concentration, it can therefore be inferred that both of these metrics also scale with true OCR, barring any diffusion limitations or other such effects. As such, any index computed from these values could be considered a "true" index, because the scaling factor would cancel with the calculation of the ratio. The results empirically demonstrated all of the above necessary validation. Further, the ratio of dO_2 slope in high glucose to that in low glucose (i.e., the dO_2 slope index) was highly predictive of the length of time to achievement of normoglycemia in transplanted mice.

MATERIALS AND METHODS

Islet Isolation and Culture

Islets were isolated using a modified version of the automated method described previously (23) and were allowed to recover for 24–72 h at 37°C in conventional islet medium (Cell-Gro, Cat. # 99-663-CV) before transplantation and OCR assessment. On the day of OCR measurement and transplantation, islet equivalents (IEQs) were counted based on conventional dithizone staining using an inverted stereomicroscope with a reticule. Except where noted, all experiments were conducted using 500 IEQ per well.

Oxygen Consumption Measurements: Plate Setup

The principles of the OBS system (BD Biosciences Cat. # 353830) have been described previously (8,33). Briefly, a standard round-bottom 96-well microplate is coated with a suspension of silicone rubber containing a ruthenium-based oxygen-sensitive fluorophore, the intensity of which varies inversely with the oxygen concentration at the well bottom.

OBS measurements were taken using a temperature-controlled fluorometric plate reader (Perseptive Biosystems, Cytofluor II) set to excitation 480 ± 20 nm and emission 620 ± 40 nm, with a signal gain of 40. Instrument temperature was maintained at 37 ± 0.1°C to en-

sure a stable baseline fluorescent signal. Empty plates were allowed to warm for 30 min in the fluorometer prior to seeding. Then plates were read empty to normalize fluorescence values by well, as described below. These settings and practices are paramount to collecting the data used for these analyses.

Samples from each islet preparation were divided into two approximately equal aliquots, pipetted into two 1.5-ml microfuge tubes, and pelleted by a pulse spin for 10 s in a microfuge at 1000 rpm ($82 \times g$). Culture medium supernatant was aspirated and replaced with a predetermined volume, based on IEQ number, of 37°C Krebs buffer (without bicarbonate, buffered with 25 mM HEPES and adjusted to pH 7.4) containing either low (2.2 mM) or high (22.2 mM) glucose.

Eight wells of the plate were filled with 200 μl of prewarmed Krebs buffer without cells as ambient dO_2 controls. Another eight wells were filled with 200 μl of 100 mM sodium sulfite dissolved in double-distilled sterile water as anoxic controls. These two control conditions serve as a two-point in situ calibration and are essential for fluorescence signal normalization (as described below). Each test condition (high glucose and low glucose) was set up in triplicate. The liquid volume in all test wells was 200 μl. Eight wells were left empty to follow sensor drift, which was negligible.

Cells and control conditions were added to the prewarmed (37°C) plate within 4 min to minimize cooling. The plate was placed in the temperature-controlled plate reader and read at 5–6-min intervals at 37°C for 200 cycles. Upon completion, the cells were collected into 1.5-ml microfuge tubes by aspiration. An inverted microscope was used to ensure that all cells and debris were aspirated. The aspirates were pulse-spun in a microcentrifuge to pellet the tube contents. The supernatant was aspirated and 1 ml of AT-Extraction solution (0.066 N ammonium hydroxide/0.002% Triton X-100) was added to each tube to solubilize the tissue for later DNA analysis.

DNA Assessment/Pico Green Method

Human dsDNA standards (Sigma) were prepared in Tris-EDTA buffer by serial dilution of a 37.5 μg/ml (1 ml final volume) frozen aliquot. Duplicates of standard values and triplicates of each test sample in extraction buffer were prepared based on initial undiluted readings, neat or diluted. For all samples 100 μl was pipetted into a 96-well flat-bottom polystyrene plate (Costar) and 100 μl of Pico Green dsDNA probe (Molecular Probes Eugene, OR), diluted 200 times in Tris-EDTA buffer, was added to each well. The plate was placed in a fluorometer (Perseptive Biosystems, Cytofluor II) and incubated for 5 min without exposure to light. The plate was then read at an excitation of 485 ± 20 nm and emission of 530 ± 20 nm at a gain setting of 80. DNA was quantified against the standard curve using standard methodology.

Calculation of dO_2

Values for dO_2 were computed from the fluorescence data as previously described (7). Raw fluorescence values for each well were normalized to their "blank" reads, yielding an adjusted intensity (I′) to correct for any variations in fluorescence intensity due to real or instrument-perceived variations in the concentration of fluorophore in each well. At each time point, the I′ values were then normalized to the mean I′ value of the eight media-containing wells, resulting in a normalized fluorescence value, N, which represents the relative fold increase in signal in experimental wells relative to negative control wells. This second normalization removes any drift in signal due to fluctuations in temperature, excitation light intensity, etc., and also defines the ambient controls as having an N of 1.00. Unprocessed data were inspected to make sure the negative control wells showed no significant change in fluorescence. Figure 1a shows a representative plot of N versus time for one preparation.

From the normalized fluorescence values, the oxygen concentration in each well at every time point measured was calculated using the following manipulation of the Stern-Volmer equation:

$$dO_2 = (DR/N - 1)/K_{SV} \quad (1)$$

DR, the dynamic range of the plate, is the maximum possible increase in signal as determined from the average value of N for the fully deoxygenated sodium sulfite controls. K_{SV} is the Stern-Volmer constant, equal to $(DR - 1)/O_a$, where O_a is the ambient oxygen concentration, which we assume constant at 210 μM. Figure 1b shows dO_2 versus time for the same representative example as in Figure 1a. All the dO_2 plots exhibited a shape similar to that shown in Figure 1b, with an initial, empirically linear decrease followed by a transition to a fairly constant steady-state value.

OCR Estimates

Two different estimates of OCR were calculated from the oxygen concentration data. One derives OCR from the difference at equilibrium between ambient dO_2 and the measured dO_2, via the steady-state solution to Fick's Law (7). Here, the assumption is made that the cells are homogenously distributed along the well bottom and that there is no significant effect of convection or diffusion through the walls of the well. The other method is based upon the empirical observation that measured dO_2 decreased empirically/linearly during the transient phase following seeding, and that the rate scaled linearly with cell number/DNA concentration. This was first observed

with hepatocytes (35), and the present data show that it also applies to islets.

Per the equilibrium Fickian model, the rate of oxygen diffusion into the well at any time point will be equal to the OCR of the cells, and directly proportional to magnitude of the dO_2 gradient (8,17). Under the conditions employed, the theoretical maximal oxygen transfer rate (OTR) into the plate wells is 2.0E+05 fmol min^{-1} (7). The OCR was thus computed as this maximal rate multiplied by the fractional decrease in dO_2 observed, as given by the following equation:

$$OCR = (O_a - O_t)/O_a * OTR_{max} \quad (2)$$

O_t is the value of dO_2 at time *t* computed via equation (1). This model is based upon a number of simplifying assumptions. The potential impact on this model of the non-idealities present in the actual system have been previously discussed (7).

The rapid method estimates OCR from the initial rate of change of measured dO_2 expressed in μM min^{-1}. This value was calculated as the slope of a least-squares fit to the computed values of dO_2 between 20 and 60 min. This time window was adjusted as necessary to ensure that only the empirically linear portion of the dO_2 trace was being fit.

OCR Index

OCR estimates were determined by both methods described above and then normalized for corresponding DNA concentrations from the same well. The triplicate high-glucose OCR values were averaged and divided by the average low-glucose values, giving a final OCR index.

Nutrient Deprivation/Hypoxia Experiments

To confirm whether damage or injury to islets is reflected in the OCR and/or the OCR index, an aliquot from three preparations was subjected to oxygen limitation to compromise the overall health of the islets. Aliquots of 3,000 human IEQs were utilized for each condition, control, and pellet. Control islets were cultured for 3 h (one preparation) or 6 h (two preparations) in 37.5-mm petri dishes (Costar) in standard culture conditions. Test islets were subjected to hypoxic conditions by pelleting the cells to the bottom of a 15-ml conical tube containing 15 ml of standard culture medium and storing them with the cap on throughout the culture period. A fluorescence-based needle oxygen probe placed 1 mm above the surface of a 3,000 IEQ pellet for 16 h confirmed that within 75 min the oxygen concentration at the needle sensor measured 0 mmHg, and this reading was maintained for the duration of the reading (data not shown). This approach has proven a more effective means of inducing cell dysfunction and death than the use of chemical insults, and generates highly reproducible differences between the abused cells and their matched controls (12).

In Vivo Assessment of Islet Function

Approved protocols (Institutional Animal Care and Use Committee were executed in the Preclinical Cell Processing Core of the Cell Transplant Center at the Diabetes Research Institute. Male athymic *nu/nu* (nude) mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed in virus- and antigen-free rooms in micro-isolated cages at the Division of Veterinary Resources of the University of Miami. Animals were rendered diabetic via a single intravenous administration of 200 mg/kg of streptozotocin (Sigma-Aldrich, St. Louis, MO). Nonfasting blood glucose was assessed by glucometer (Elite, Bayer, Tarrytown, NY) and mice with sustained hyperglycemia (>300 mg/dl) were designated for islet transplant. Grafts (2,000 IEQ per recipient) were transplanted under the kidney capsule (1–3 per preparation) using recipients matched for body weight similarities. Graft size was matched by counting and pellet volume. For impure islet preparations, the cells were transplanted across both sides of the kidney to avoid oxygen limitations caused by contaminating tissue mass. After transplantation, nonfasting blood glucose values were assessed daily for the first week and then three times a week following for 60 days. Reversal of diabetes was defined as stable nonfasting blood glucose <200 mg/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 and maintaining normoglycemia after transplantation.

Statistical Analysis

All statistical analyses reported were generated using the Open Source statistics software R V2.1.0 along with the packages "survival" V2.18 and "rpart" V3.1-23 on a combination of RedHat Linux and Windows XP platforms.

Logistic Regression Analysis. Once the correlation between dO_2 slope, equilibrium OCR, and cell number/DNA concentration was established, only dO_2 slope was further used as a metric. For the purposes of establishing a potency metric, the "input" data statistically analyzed consisted of dO_2 slopes in both low and high glucose (raw values and DNA normalized) and the corresponding index values (ratios of dO_2 slope in high vs. low glucose). The objective of this analysis was to determine the ability of these readings to predict the "output" data: the time to cure for 69 mice distributed among 27 islet preparations/treatments.

Recursive Partitioning Analysis. In order to determine optimal cut-off criteria for predicting the time to cure, a recursive partitioning analysis was carried out using the R function "rpart" for each of the five parameters as dependent variable and the days to cure as the independent variable. Recursive partition analysis is a common statistical tool for partitioning data into groups by systematically assessing each predictor and all potential cutpoints. Partitioning is optimized by minimizing false-negative and false-positive instances for the outcome variable at each step. Partitioning is repeated until any of the subgroups contain a homogenous group or the subgroups are too small for further subdivision (6).

Kaplan-Meier Survival Analysis. As a final component of the analysis, a standard Kaplan-Meier type survival analysis was conducted using the cutoffs specified from the partitioning analysis. *p*-Values were calculated using a log-rank test.

RESULTS

Establishment of OCR Methodology

Normalized fluorescent values, N, for triplicates of 500 IEQ in low- and high-glucose KRB from one preparation (HP) are given in Figure 1a; corresponding values of dO_2 computed per equation (1) are in Figure 1b. The triplicates shown are reproducible and the high- and low-glucose curves are clearly resolved from each other.

Figure 2 plots the initial rate of decrease of dO_2 ("dO_2 slope") as a function of DNA concentration for preparation HP 1. It can be seen that for either glucose concentration, the dO_2 slope scales empirically linear with DNA (cell number), but that the dO_2 slope is consistently higher for the higher glucose concentration. Other preparations for which a range of IEQ numbers and therefore a range of DNA concentrations were plated show a similar trend. This is consistent with trends previously observed with primary rat hepatocytes (3).

Based on these results, it was deemed that a target of 500 IEQ per well was optimal—large enough to minimize signal-to-noise issues, while small enough to avoid immediate oxygen starvation. The data in Figure 2 also point out that the amount of DNA extracted from the various wells was not the same, despite attempts to aliquot comparable IEQs to each well. Given the inherent limitations of IEQ counting and the difficulties in pipetting a precise number of clustered cells to any given well, it was also deemed best to normalize all estimates of OCR to cell number, by way of total DNA content.

Figure 3 shows a similar comparison for the equilibrium OCR estimates. In some cases the equilibrium OCR values are high enough (equilibrium dO_2 values low enough) that it is likely that the cells are diffusion limited for oxygen. One would expect to see a deviation from linearity under these conditions (7). A plot of all measured values of dO_2 slope versus all measured equilibrium OCR values (Fig. 4a) is consistent with this expectation. The variability in the numbers is likely a combination of experimental error and the fact that for diversely sized cell clusters such as islets, internal oxygen diffusion limitations would become relevant before the environment at the well bottom became completely anoxic. This plot demonstrates that the initial slope method enables us to estimate OCR at high cell densities that would become diffusion limited if applying the equilibrium method.

For any given sample, we are confident that the initial slope correlates to the OCR and, thus, to the OCR index. Work investigating whether the dO_2 slope correlates universally to cell number, DNA concentration, and other means of measuring OCR is ongoing.

Correlation of OCR Results to Mouse Transplantation Results

Results from the 27 human islet preparations assessed in this study are outlined in Table 1. Raw and DNA-normalized initial rates of change of measured dO_2 ("dO_2 slope") in both low- and high-glucose medium are shown, as well as the computed dO_2 slope indices and the time to reversal of hyperglycemia in each transplanted animal. It can be seen that the computed values of dO_2 slope spanned approximately two orders of magnitude. Indices ranged from 0.78 to 2.4, and the time to reversal (for animals that reversed) ranged from 1 day to 33 days.

Diabetes was ultimately reversed in the majority of mice transplanted with islets that did not undergo purposeful damage via hypoxia. The islets subjected to hypoxia (HP 5A, 6A, 9A) consistently produced OCR values in both low and high glucose that were depressed by 30–50% compared to matched controls. Poor function in these damaged islets was confirmed by a low dO_2 index (less than the cutoff value described below) and the observation that none of the mice transplanted with the damaged islets achieved reversal of their diabetes. In those preparations not damaged by hypoxia, only three had OCR indices less than 1.0, and those preparations had the longest times to reversal.

Our data suggest that OCR alone is not an accurate predictor of islet function in vivo after transplantation, regardless of whether the measurement was taken in high- or low-glucose Krebs solution. Figure 5, a plot of the high dO_2 slope normalized for DNA versus the percentage of animals cured, demonstrates a predictive capacity of the high-glucose dO_2 slope alone, yet an inability of this value to distinguish between fast and slow

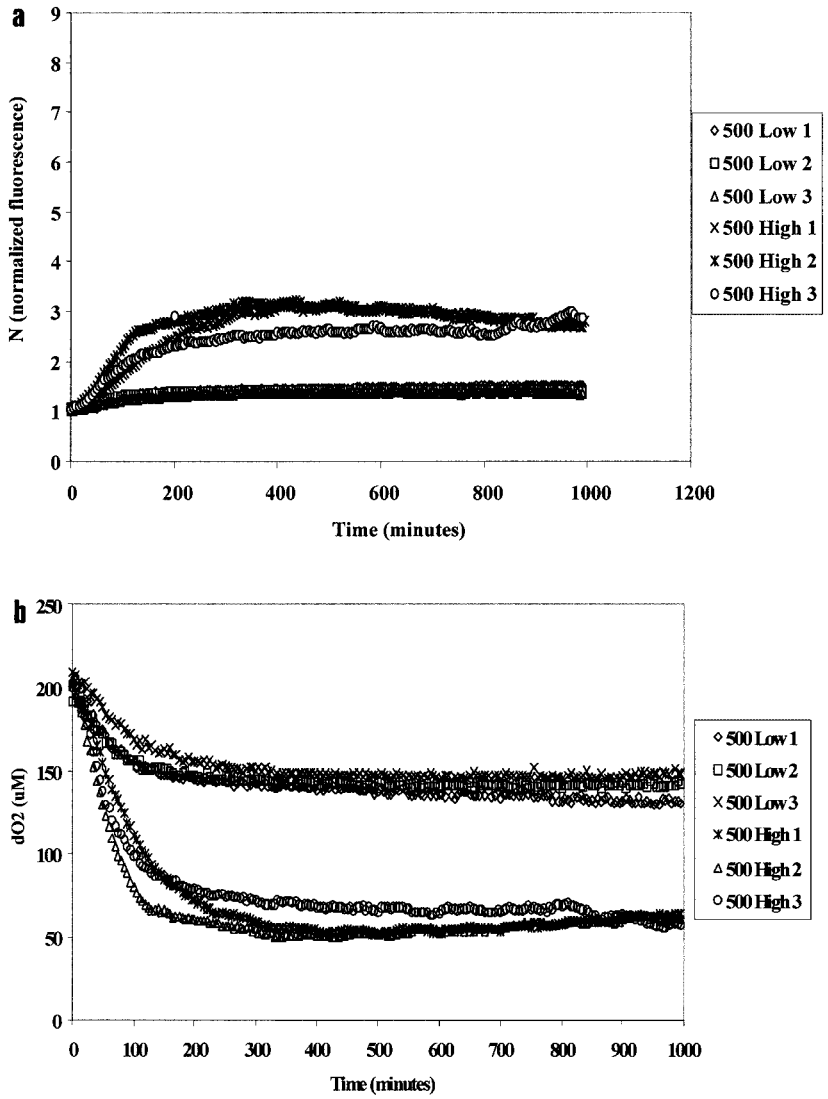

Figure 1. Representative plots versus time for a single islet preparation (HP 24) of (a) normalized fluorescence, N, and (b) computed dO_2.

curing preparations. In contrast, the dO_2 slope index proved a more accurate predictor of the time to cure, and, thus, of the IEQ potency. Figure 6 shows the dO_2 slope versus the corresponding index for each preparation. This figure shows that a high dO_2 slope is not directly related to time to reversal and therefore not correlated with potency. Several preparations with an elevated dO_2 slope in high glucose failed to reverse by the optimal cutoff point of 3 days and had indices close to the cutoff point. Conversely, other preparations had lower high-glucose dO_2 slope values but substantially higher indices. When transplanted all these preparations produced reversals within 3 days.

Statistical Analysis: Establishment of Best Parameters and a Cutoff Ratio to Predict Cure

Relationship Between Initial and EQ Measurements. The primary focus of this analysis is on the initial slope method, but a brief comparison is made to equilibrium OCR (EQ). Of the 27 human islet preparations, 16 had equilibrium OCR readings at or near the maximum value for the system, indicating these conditions were diffusion limited for oxygen. As such, the equilibrium OCR readings for these preparations were artificially low and not used in any subsequent analysis. For the 11 islet preparations whose equilibrium readings were "on

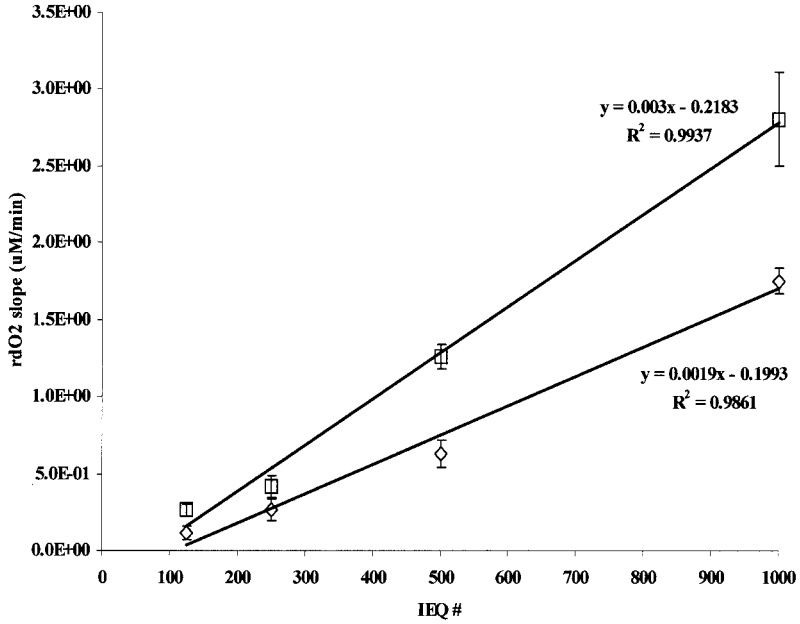

Figure 2. Plot of rdO_2 slope as a function of number of IEQ seeded per well, for low (diamonds) and high (squares) glucose. Linear least-squares fits to the data points are shown. Error bars represent SD.

scale," a linear regression was performed between the initial and equilibrium readings, and the correlation and R^2 values were determined. The results are displayed in Table 2. All subsequent analysis was done with the dO_2 slope readings only and included all preps.

Logistic Regressions. The independent variable utilized in the logistic regression analysis was the time to cure and the dependent variables were the log ranks of the five parameters. The *p*-value plots for this analysis are shown in Figure 1. Lower *p*-values correspond to

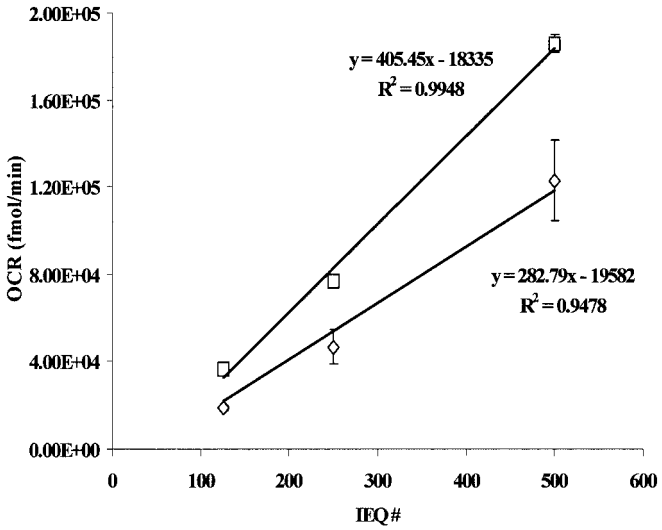

Figure 3. Equilibrium OCR as a function of IEQ number for low (diamonds) and high (squares) glucose. Linear least-squares fits to the data points are shown. Error bars represent SD.

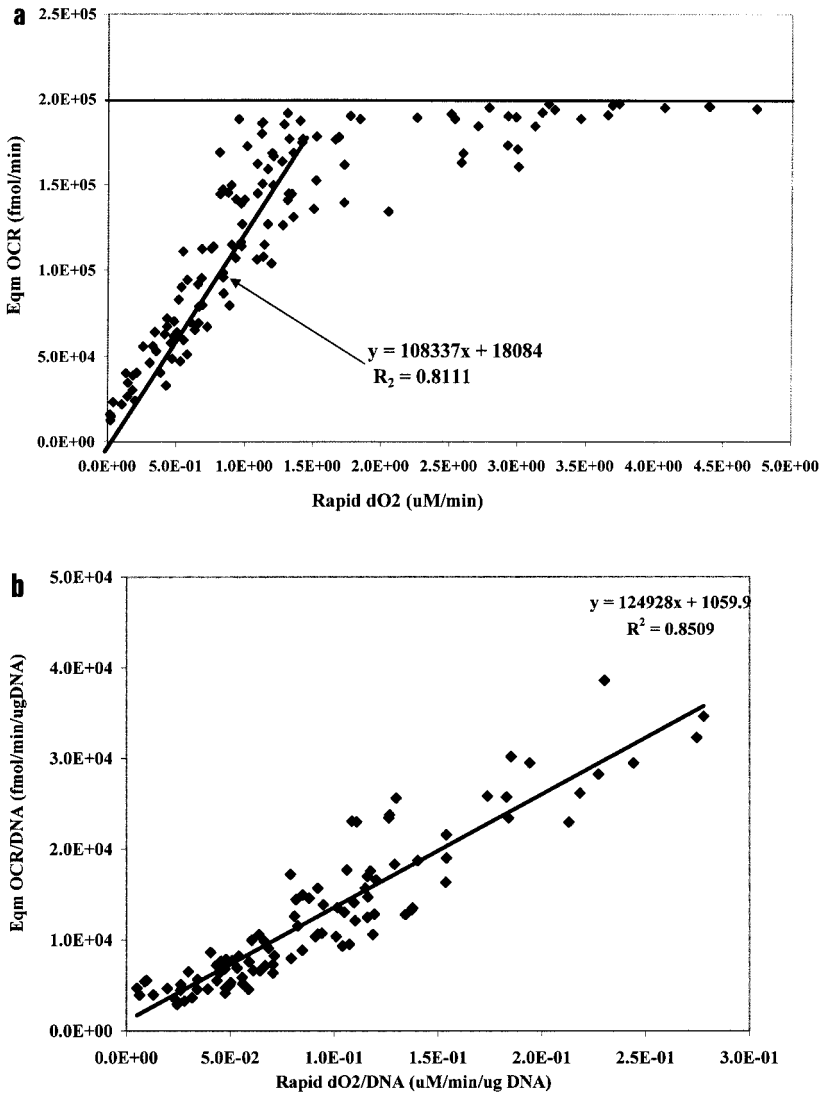

Figure 4. rdO_2 as a function of equilibrium OCR. (a) Raw readings, not DNA normalized, across the 27 preparations. The linear least-squares fit to the data considers only those 14 preparations for which the equilibrium OCR was not diffusion limited. (b) DNA-normalized values from the 14 non-diffusion-limited conditions.

better predictive capacity. With the exception of the DNA-normalized high-glucose dO_2 slope, dO_2 slope readings by themselves were not predictive of cure rate. Of the five parameters examined, only the dO_2 slope index had a *p*-value less than 0.01. This analysis establishes that the dO_2 slope index is the best predictor of time to cure.

Recursive Partitioning Analysis. Figure 8 shows that the optimal partitioning divided the population into a two sets: one of 43 mice with an dO_2 slope index ≥1.27, mean cure time of 2.7 days, and a 2× mean standard error of 5.0 and another of 26 mice with an dO_2 slope index <1.27, mean cure time of 26.9 days, and a 2× mean standard error of 12.4. It should be noted that there were two obvious outliers, one in each population. There was one instance (HP 7) where the index was slightly above the cutoff, yet one animal took 33 days to reverse. During the renal subcapsular procedure, there was a technical problem and some of the islets may have leaked out of the capsule of this particular animal, which could explain the delayed reversal. A number of experi-

mental errors of this sort likely contribute to the variability in the observed data. In another case (HP 15), there was one animal that reversed immediately despite a less than optimal dO_2 index (1.17).

Kaplan-Meier Analysis. A typical survival plot is shown in Figure 9. The top curve in each plot is the "cure" curve for the mice with OCR index values above the cutoff value, and the bottom curve is for mice with values below the cutoff. The dashed lines about each curve represent 95% confidence intervals for the fraction cured. A log rank test was conducted to compare the two survival curves and they were significantly different with $p < 0.00001$. These data support the claim that it is possible to utilize these methods to discriminate between cells that are highly likely to effect a rapid cure and cells that may be delayed in providing a cure or provide no cure at all.

DISCUSSION

The empirical demonstration that the slope of the dO_2 signal during the transient phase reproducibly correlates with the steady-state OCR and cell number (DNA) allows it to be used as a more rapid assessment of OCR. Comparison of these OCR estimates to absolute OCR numbers derived from more traditional methods is in progress.

The data presented demonstrate a strong correlation between the DNA-normalized dO_2 slope index and time

Table 1. Summary of Results of Analysis of 27 Human Islet Preparations

Prep	rdO_2 Low (μM/min μg DNA)	rdO_2 High (μM/min μg DNA)	rdO_2 Index	OCR Low (fmol/min μg DNA)	OCR High (fmol/min μg DNA)	Purity	Tx	Cured	Cure Rate	Cure Day
HP 1	0.098 ± 0.017	0.15 ± 0.061	1.60	1.2E+04 ± 4.6E+03	2.1E+04 ± 6.2E+03	90	3	3	1.00	2, 2, 1
HP 2	0.12 ± 0.0097	0.15 ± 0.057	1.30	1.5E+04 ± 7.9E+03	2.5E+04 ± 1.7E+03	90	3	3	1.00	1, 1, 1
HP 3	0.17 ± 0.06	0.13 ± 0.021	0.78	1.2E+04 ± 2.0E+03	9.6E+03 ± 4.2E+02	50	4	1	0.25	17
HP 4	0.094 ± 0.023	0.15 ± 0.051	1.64	1.2E+04 ± 3.5E+03	1.9E+04 ± 3.3E+03	90	3	3	1.00	1, 1, 1
HP 5	0.093 ± 0.027	0.20 ± 0.020	2.20	1.4E+04 ± 1.7E+03	2.4E+04 ± 2.5E+03	80	3	3	1.00	6, 1, 1
HP 5A	0.054 ± 0.013	0.049 ± 0.0039	0.90	6.8E+03 ± 2.1E+03	7.0E+03 ± 2.9E+02	80	3	0	0.00	N/A
HP 6	0.14 ± 0.047	0.14 ± 0.018	0.95	1.1E+04 ± 4.9E+03	9.9E+03 ± 9.9E+02	95	3	1	0.33	6
HP 6A	0.038 ± 0.012	0.037 ± 0.0094	0.98	3.9E+03 ± 7.8E+02	4.4E+03 ± 8.4E+02	95	3	0	0.00	N/A
HP 7	0.64 ± 0.15	0.83 ± 0.37	1.30	4.3E+04 ± 5.2E+03	4.0E+04 ± 1.6E+04	80	2	2	1.00	1, 33
HP 8	0.38 ± 0.058	0.64 ± 0.019	1.69	2.3E+04 ± 1.8E+03	2.3E+04 ± 5.0E+02	80	3	3	1.00	1, 2, 2
HP 9	0.30 ± 0.15	0.42 ± 0.21	1.42	2.7E+04 ± 1.2E+03	3.1E+04 ± 3.6E+03	90	3	3	1.00	5, 3, 7
HP 9A	0.13 ± 0.0056	0.16 ± 0.061	1.25	1.3E+04 ± 3.5E+02	1.7E+04 ± 1.0E+03	90	3	0	0.00	N/A
HP 10	0.05 ± 0.0061	0.11 ± 0.015	2.24	7.7E+03 ± 5.1E+02	1.4E+04 ± 1.8E+03	90	2	2	1.00	1, 1
HP 11	0.18 ± 0.014	0.22 ± 0.025	1.20	3.8E+04 ± 1.7E+04	4.5E+04 ± 1.1E+04	90	1	1	1.00	12
HP 12	0.055 ± 0.0026	0.056 ± 0.0079	1.02	3.9E+03 ± 2.2E+03	2.9E+03 ± 9.7E+02	80	3	3	1.00	11, 25, 8
HP 13	0.070 ± 0.0074	0.12 ± 0.0089	1.79	9.9E+03 ± 1.4E+03	1.3E+04 ± 9.3E+02	95	1	1	1.00	1
HP 14	0.089 ± 0.011	0.13 ± 0.013	1.43	6.3E+03 ± 1.9E+02	8.6E+03 ± 1.6E+03	75	1	1	1.00	1
HP 15	0.086 ± 0.0079	0.10 ± 0.0074	1.17	4.8E+03 ± 1.1E+02	6.6E+03 ± 1.2E+03	85	2	2	1.00	1, 6
HP 16	0.068 ± 0.0029	0.070 ± 0.0047	1.02	8.6E+03 ± 1.8E+03	8.6E+03 ± 5.7E+03	95	1	1	1.00	7
HP 17	0.062 ± 0.006	0.065 ± 0.0023	1.04	3.2E+03 ± 2.6E+02	2.1E+03 ± 2.0E+02	50	3	0	0.00	N/A
HP 18	0.15 ± 0.020	0.20 ± 0.020	1.66	1.7E+04 ± 3.0E+03	2.2E+04 ± 8.2E+02	60	3	3	1.00	1, 1, 1
HP 19	0.40 ± 0.020	0.52 ± 0.097	1.32	2.1E+04 ± 9.4E+02	2.3E+04 ± 3.3E+03	80	1	1	1.00	1
HP 20	0.13 ± 0.027	0.18 ± 0.063	1.39	9.4E+03 ± 1.0E+03	1.2E+04 ± 6.8E+03	95	2	2	1.00	1, 1
HP 21	0.073 ± 0.013	0.14 ± 0.015	1.92	1.3E+04 ± 2.7E+03	1.7E+04 ± 4.0E+03	90	2	2	1.00	1, 1
HP 22	0.11 ± 0.039	0.15 ± 0.011	1.40	5.2E+03 ± 1.4E+03	6.6E+03 ± 3.7E+02	40	1	1	1.00	12
HP 23	0.077 ± 0.041	0.14 ± 0.077	1.81	1.3E+04 ± 6.9E+03	2.2E+04 ± 1.0E+04	90	1	1	1.00	1
HP 24	0.056 ± 0.012	0.084 ± 0.016	1.51	6.0E+03 ± 1.1E+03	9.3E+03 ± 3.8E+03	95	2	2	1.00	3, 3
HP 25	0.13 ± 0.037	0.23 ± 0.085	1.81	1.7E+04 ± 3.6E+03	1.8E+04 ± 2.8E+03	80	2	2	1.00	1, 1
HP 26	0.025 ± 0.009	0.039 ± 0.0053	1.57	4.0E+03 ± 6.7E+02	7.1E+03 ± 2.1E+03	90	2	2	1.00	1, 1
HP 27	0.30 ± 0.045	0.44 ± 0.063	1.49	4.7E+04 ± 6.4E+03	5.0E+04 ± 6.7+03	90	3	3	1.00	1, 1, 1

Preparations indicated with "A" following the HP number are IEQs from the same preparation subjected to hypoxia/nutrient starvation. Tx refers to number of animals transplanted and cured refers to animals where normoglycemia was achieved. The purity was determined using conventional dithizone staining and assessment by trained cGMP personnel.

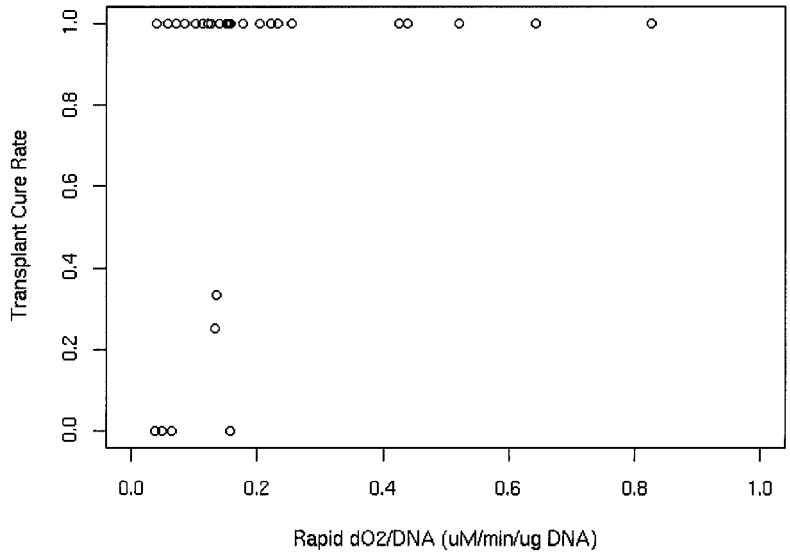

Figure 5. Cure rate as a function of high glucose rdO_2.

to cure in concurrently transplanted nude mice. While OCR has been demonstrated by others as an effective measure of islet potency for pretransplant assessment (21), the ease of use of the BD OBS offers significant advantages over traditional methods. There are several technical considerations in the collection of OCR index data, however, that will play a critical role in the ability of the end-user to make accurate predictions regarding islet potency.

The choice of medium utilized for assessment is one

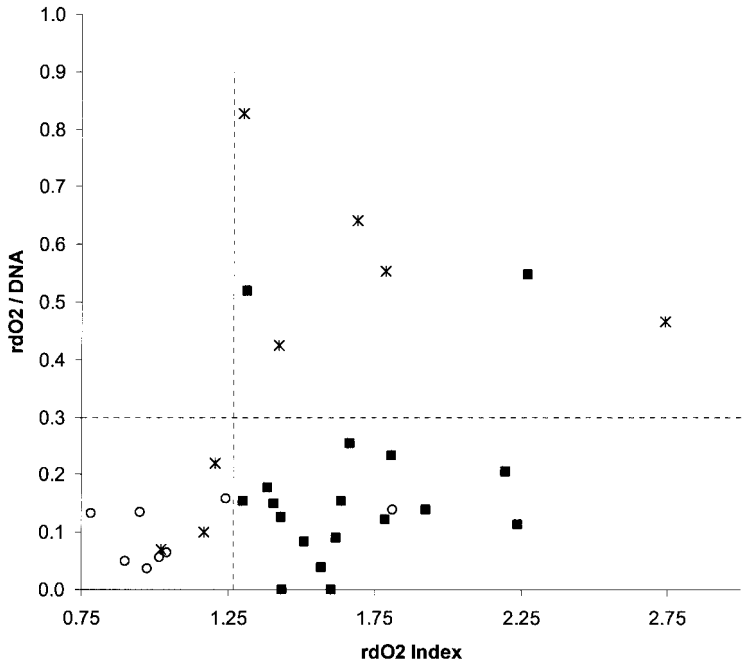

Figure 6. Transplant effectiveness as a function of both the OCR index and the DNA-normalized rdO_2 value. Indicated are preparations for which at least one of the replicate transplantations failed to reverse (circles), preparations where all replicates reversed within 3 days (X), and preparations where all animals reversed, but not all within 3 days (squares).

Table 2. Correlation of dO_2 Slope and Equilibrium OCR Measurements in 11 Human Islet Preparations Where Equilibrium Measurements Were on Scale and Not Diffusion Limited

Glucose	Reading	R	R^2	p-Value
Low	dO_2, EQ OCR	0.79	62.4%	0.0008
Low	dO_2/DNA, EQ OCR/DNA	0.90	81.4%	<0.0001
High	dO_2, EQ OCR	0.80	63.8%	0.0006
High	dO_2/DNA, EQ OCR/DNA	0.91	82.1%	<0.0001
High/low	ratio	0.62	39.0%	0.0169

consideration. The literature suggests glucose-responsive changes in insulin release and OCR can be detected reliably in minimal media such as Krebs solution (9), but nutrient-rich media can reduce the ability to detect glucose-responsive OCR changes (2,29,33). It is our assertion that such glucose-responsive changes are critical to the assessment of islet potency. Historical data show that measurements taken from islets (including insulin secretion and OCR) are more meaningful when differential concentrations of glucose are utilized, due to the clear association of glucose responsiveness of islets with islet function (16,25).

Purity is another consideration important in evaluating islet preparations. The ideal situation would be to transplant and analyze the OCR of pure islets. However, because islets constitute less than 2% of the pancreas, virtually all clinical islet preparations contain both exocrine and ductal cells, despite purification, which will impact both the measured OCR per cell and the effective glucose responsiveness of the tissue sample. In 18 of the 27 preparations evaluated, islet purity was estimated at 90% or greater based on standard dithizone staining and microscopic evaluation; 22 of 27 were 80% or greater. Among the preparations of low purity, HP 3 (purity 50%) had the lowest OCR index measured and only one out of four animals transplanted reversed diabetes (at day 17). Despite very low purity (40%), HP 22 had an index of 1.4, and did provide a cure to the mouse transplanted, although delayed (12 days). The index would likely have been higher in this preparation had the purity been greater.

The exocrine cells that often contaminate human islet preps can clearly contribute to OCR. However, others have demonstrated that there are no changes in the OCR of exocrine cells in response to changes in glucose concentration (21). As such, the measured OCR index is a function of purity. While further experiments are needed to clarify the effects of purity on OCR index and subsequent transplant outcome, it should be pointed out that exclusion of the low-purity cases from Figure 6 does not substantively change the trends in the data. It may ultimately be desirable in practice to adjust the mass of tissue transplanted based on a number of in vitro metrics, including purity and OCR index.

The present data set includes five preparations for which index values less than unity (1.0) were computed, which seems counterintuitive. The explanation may lay

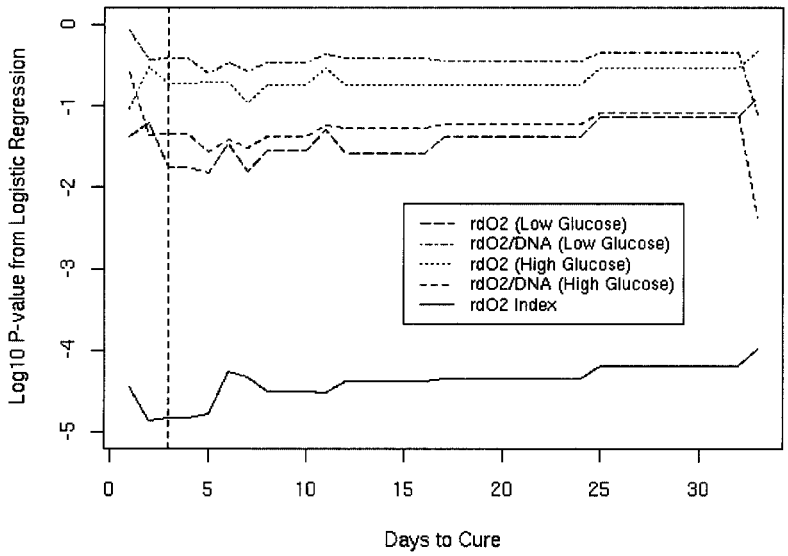

Figure 7. Logistic analysis to determine the best predictor of the time to cure.

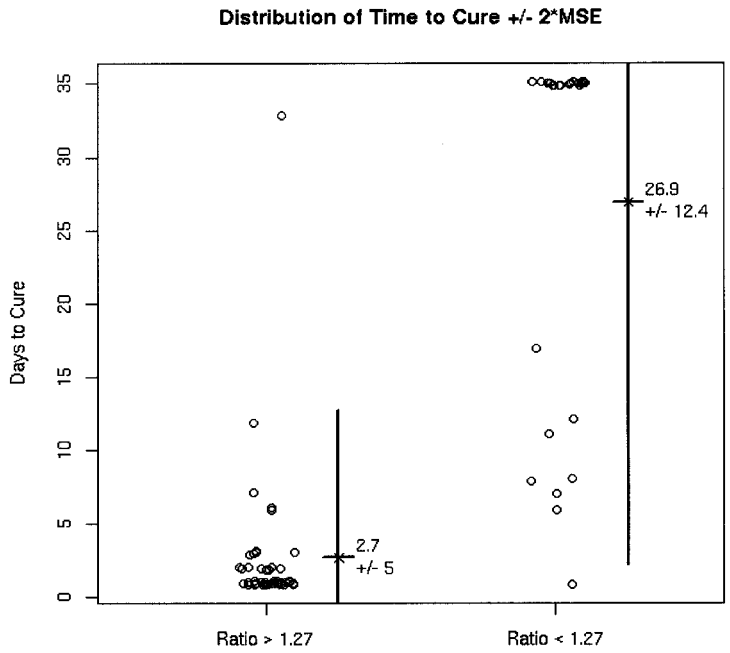

Figure 8. Establishment of a cutoff ratio for prediction of islet transplant outcome.

at least partially in the fact that the index is computed from six separate experimental numbers (OCR per DNA at each of two glucose concentrations). As such, the experimental uncertainty in the index number can be fairly high.

Experimental errors notwithstanding, it cannot be ruled out that some cells truly had index values less than unity. It is conceivable that some combination of apoptosis-induced OCR increase and glucose-induced toxicity resulted in a condition whereby the cells actually exhibited a higher OCR per cell in low glucose. More experiments purposefully introducing mitochondrial stresses would need to be performed to confirm this hypothesis, which has been demonstrated with other cell types in similar studies (11,22).

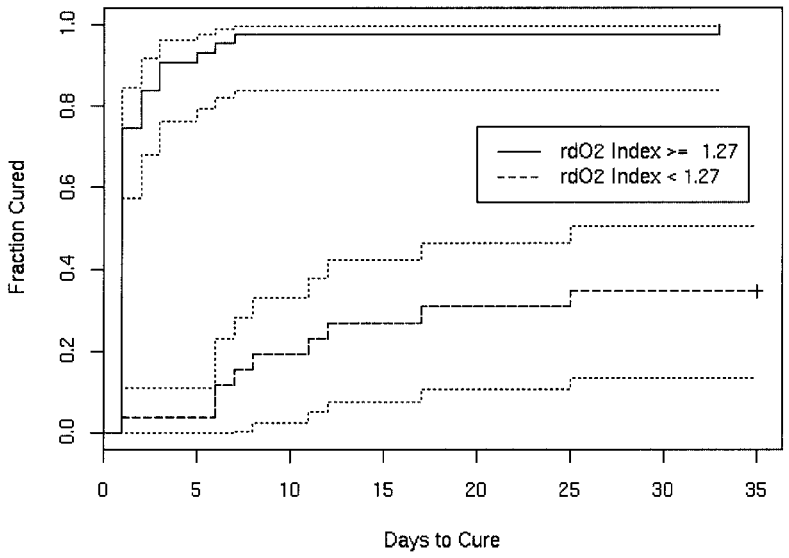

Figure 9. Survival analysis. Kaplan-Meier survival plot showing fraction of transplanted mice cured by a given day for the subpopulations with an rdO_2 index below or above the cutoff value of 1.27. The fine dotted lines represent 95% confidence intervals.

In summary, the data presented herein strongly suggest that OCR alone is not a sufficiently accurate predictor of islet function. Only when the islets were subjected to significant hypoxic damage did we observe a significant depression in the OCR in both high and low glucose when compared to uncompromised matched controls. Our studies demonstrate that the ratio of OCR in high- versus low-glucose media most accurately describes the state-of-function of the islets. For preparations where all transplanted animals reversed within 3 days, the OCR index universally exceeded the determined cutoff value of 1.27. For all preparations where at least one animal failed to reverse at all, index values were universally less than 1.27. Relying solely on OCR without considering glucose responsiveness may lead to false assumptions about the potency of the islets.

Because murine renal subcapsular transplants of human islets show some correlation to the clinical outcome of human transplants of islets from the same preparation, the present results make it reasonable to hypothesize that the same predictive ability could be achieved through the use of the BD OBS and the methods described herein (10). Further work investigating the utility of the OBS as a potency assay is ongoing.

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