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Use of integrin-linked kinase to extend function of encapsulated pancreatic tissue

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Abstract

We have studied the impact of overexpression of an intracellular signaling protein, integrin-linked kinase (ILK), on the survival and function of encapsulated islet tissue used for the treatment of type 1 diabetes. The dimensions of the encapsulated tissue can impact the stresses placed on the tissue and ILK overexpression shows the ability to extend function of dissociated cells as well as intact islets. These results suggest that lost cell–extracellular matrix interactions in cell encapsulation systems can lead to decreased insulin secretion and ILK signaling is a target to overcome this phenomenon.

1. Introduction

Transplantation of encapsulated insulin-producing islets of Langerhans for the treatment of type 1 diabetes has been a topic of research for decades (Lim and Sun 1980). Despite the considerable efforts made worldwide, an effective strategy to maintain the long-term viability and function of transplanted tissue has remained elusive. A successful system needs to allow the islet tissue to maintain critical functions as it did in the pancreas. For encapsulation systems, the minimal requirements include: access to nutrients, removal of metabolic waste and proper interactions with extracellular matrix (ECM)-binding moieties among others.

A negative consequence of the encapsulation procedure is the prevention of revascularization of encapsulated islets, which presents a major hurdle for long-term function. The isolation of islets within a capsule can lead to hypoxic stress due to the high metabolic activity of islet cells and the longer diffusion distance for oxygen to reach the cells. Studies using rat islets showed that even under normoxic conditions (20% O₂), the central cells in larger islets can undergo apoptosis due to diffusion-limited access to nutrients like oxygen (Moritz *et al* 2002). Under hypoxic conditions (1% O₂) central apoptosis was noticeable after 6 h with almost complete loss of viability in the core after 48

h. Studies have shown that islet size influences graft performance and one strategy to extend function involves dissociating the native islet structure (approximately 150–200 μm in diameter) into single cells or clusters of cells (Giuliani *et al* 2005, Lehmann *et al* 2007) to facilitate the availability of oxygen and other important nutrients.

While the problem of hypoxia motivates the creation of small islet tissue clusters, changes to the native islet architecture and disruption of cell–cell communication within an islet can create new problems as well (Halban *et al* 1987). Anoikis (cell death resulting from insufficient or improper cell–cell and cell–ECM interactions) is a process that has been studied primarily in relation to cancer but is applicable to the challenges of transplantation as well (Frisch and Francis 1994, Liotta and Kohn 2004, Zvibel *et al* 2002). Anoikis falls within the broader term for programmed cell death, apoptosis. Studies attempting to block apoptosis through genetic modification showed improved function for transplanted islet tissue overexpressing the anti-apoptotic protein Bcl-2 (Thomas *et al* 1999, 2001). Apoptosis can be triggered by a range of different stresses and Bcl-2 overexpression does not provide much detail about what specific stress it is blocking when function is extended.

The role of anoikis in transplanted islets has not been studied as widely as hypoxia but we hypothesize that these two stresses are inversely correlated as the tissue dimensions are varied. While smaller islet tissue clusters should be more resistant to hypoxic damage, the cells may be more susceptible to anoikis due to loss of cell–cell and cell–ECM interactions. Integrin-linked kinase (ILK) was chosen as a target to study this possible relationship and perhaps alleviate anoikis. ILK is an integrin-binding protein capable of phosphorylating Ser473 of PKB/Akt and GSK3 (Dedhar 2000, Hannigan *et al* 1996). Integrin engagement can suppress apoptosis through ILK, and ILK overexpression may reduce apoptosis through Akt and BAD phosphorylation even in the absence of integrin binding. A schematic of this pathway is shown in figure 1. Studies of transplanted hepatocytes and islets showed that RGD peptides or anti- $\beta 1$ integrin antibodies suppressed apoptosis through ILK activation (Pinske *et al* 2005, 2006). Therefore, ILK overexpression may provide some protection for transplanted cells that experience anoikis but should not impact hypoxic stress in our model.

The native islet structure has extensive connections with extracellular matrix molecules, such as laminin and type IV collagen, which are disrupted upon isolation and transplantation. Thus, we hypothesized that ILK overexpression would minimize the cellular stresses from the loss of matrix interactions. Further, as the islet is broken down into smaller clusters of cells or individual cells, a larger percentage of cells now exist in an abnormal state and the role of anoikis could increase in its importance. The encapsulation procedure prevents cell proliferation and migration so cells or cell clusters will not grow or fuse together following encapsulation. Studies were conducted both with islets isolated from mice and a murine insulinoma cell line (MIN6 cells) over-expressing ILK to determine the role of anoikis in limiting survival and function of encapsulated tissue. The MIN6 cells serve as a model for behavior of the beta cells as they are capable of glucose-stimulated insulin secretion.

2. Materials and methods

2.1. Construction of the recombinant adenoviruses

Adenoviruses containing the sequence of Cre, Bcl-2 or ILK were generated using the pAdEasy-1 system (QBiogene). These target sequences are under the control of a cytomegalovirus promoter. After insertion of the sequence, the transfer vector was allowed to undergo homologous recombination with the pAdEasy vector to produce the recombinant adenoviral genome which was linearized, transferred and amplified according to the manufacturer's specifications. Following purification of the virus, a plaque assay was

performed to determine the concentration of the viral stocks and to allow infection at specified multiplicities of infection (MOI). ILK activity was previously established by measurement of phosphorylation of protein kinase B/Akt following infection with the ILK adenovirus (Benoit *et al* 2007). The MOI of 25 used in these studies for all three viruses was based on the results of that work.

2.2. Islet isolation and cell culture

The islets used in these studies were isolated from BALB/cByJ mice at the Barbara Davis Center for Childhood Diabetes (Aurora, CO). Briefly, the pancreas was distended by injection of 3 ml of collagenase and removed from sacrificed animals. The islets were obtained by density gradient purification and hand picking using a dissection microscope. Islets were stored on ice for a period of no more than 3 h before use in our studies. All animal work and protocols were approved by the Institutional Animal Care and Use Committees at the University of Colorado and University of South Carolina.

Infections were performed prior to encapsulation for both the islets and MIN6 cells. Islets were taken off the ice used for transportation, the virus was added at a MOI of 25 in DMEM and 24 h were given for the infection to take place. MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (all from Hyclone). The virus was added at a MOI of 25 and incubated with the cells for 24 h. At the end of the infection, the cells were removed from the surface of the culture flask using 0.25% trypsin/EDTA (Hyclone) to create a cell suspension. The cell density was determined using a hemocytometer. Islets were cultured in low glucose (5 mM) RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics.

2.3. Cell encapsulation

Poly(ethylene glycol) with an average molecular weight of 10 000 Da (Sigma-Aldrich) was functionalized by addition of methacrylate end groups resulting in poly(ethylene glycol) dimethacrylate (PEGDM). Islets were allowed to settle in a microcentrifuge tube and then the media was removed. MIN6 cells were spun down in a microcentrifuge tube prior to removal of the media. The islets or MIN6 cells were then re-suspended in a solution of 10 wt% PEGDM and 0.025 wt% Irgacure 2959 which is the photoinitiator 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Ciba-Geigy) in Hank's balanced salt solution. A volume of 40 μ l was used for each resulting gel. After re-suspension of the cells, 40 μ l of the PEGDM suspension was added to a 1 ml syringe and exposed to a 365 nm light source at an intensity of 7 mW cm⁻² for 10 min under sterile conditions. Each gel in these studies contained approximately 30 islets or 50 000 dispersed MIN6 cells. The resulting gels were removed from the syringes and cultured in DMEM or RPMI 1640 on an orbital shaker set for continuous rotation at 100 rpm. Each gel was placed in separate wells of a 24-well plate in 1 ml of media. This encapsulation procedure has been shown previously to not have an adverse impact of viability for a range of cell types (Bryant and Anseth 2001, Burdick and Anseth 2002, Nuttelman *et al* 2004, Rice and Anseth 2004).

2.4. Viability analysis

Dispersed MIN6 cells or intact islets were encapsulated in PEG hydrogels and stained using a live/dead fluorescent kit (Invitrogen). Two methods were employed to quantify the percent of viable cells. For dispersed MIN6 cells, viability readings were taken on a plate reader (Biotek) over the course of 9 days with live cells identified by emission at 530 nm and dead cells by emission at 635 nm with excitation wavelengths of 480 and 530 nm, respectively. The percentage of viable cells in a sample is calculated by using the emission intensity at 530 nm (F(530)) for the sample (sam), a control with all cells viable (max) and another control with all cells dead (min). The equation is given below:

$$\% \text{Live Cells} = [F(530)_{\text{sam}} - F(530)_{\text{min}}] / [F(530)_{\text{max}} - F(530)_{\text{min}}] \times 100.$$

Confocal microscopy was also used to analyze the percentage of viable MIN6 cells following genetic modification and for all studies with islets. The encapsulation and culture conditions were identical to those listed above and the staining was performed in accordance with the manufacturer's guidelines. The studies with dispersed MIN6 cells were carried out for 9 days while intact islets were monitored for 28 days. Islet viability was measured exclusively by confocal microscopy. Results are given as a percentage of viable cells based on analysis of multiple images through the gel. Statistical analysis was performed using an unpaired Student's *t*-test.

2.5. Glucose stimulation of insulin secretion (GSIS)

Following encapsulation, GSIS was performed every 24 h to track function for up to 4 weeks. Insulin secretion was stimulated by sequential exposure of encapsulated islets to solutions with low (1.1 mM) or high (16.7 mM) glucose. The culture media was removed and replaced with 1 ml of a sterile-filtered glucose solution. After 45 min in low glucose, this solution was removed and replaced with a sterile-filtered high glucose solution. After 60 min in the high glucose solution, samples from the solution were removed and frozen for later analysis. Any remaining high glucose solution was then removed and replaced with culture media. The stimulation procedure was performed every 24 h during the duration of each experiment. The cell-laden gels were placed on the orbital shaker in the tissue culture incubator during the entire procedure.

The insulin content of each sample was measured using a high sensitivity mouse insulin ELISA kit (Mercodia). Insulin standards were used to correlate absorbance obtained from each experimental sample to the absorbance of an insulin solution of known concentration. Results were displayed relative to the amount of insulin released 1 day after the encapsulation procedure to account for variation in the amount of encapsulated tissue per gel. Viability should be close to 100% for the islets on day 1 to facilitate this normalization method.

3. Results and discussion

We investigated the impact of encapsulating MIN6 cells as dispersed single cells in PEG capsules. Analysis of MIN6 viability through live/dead staining showed a drop in viability to 20% for dispersed and encapsulated MIN6 cells over 9 days. The initial value is greater than 100% which was likely the result of having more encapsulated cells than anticipated and therefore producing a higher absorbance from the sample than the control sample of unencapsulated cells which were assumed to be 100% viable. These results are shown in figure 2. Calculation of the viable fraction of cells by microscopy removes the potential impact of varied cell number and was used for subsequent analysis. The protective effects of genetic modification were tested at the middle point in this study. Dispersed MIN6 cells were infected to overexpress ILK, the anti-apoptotic protein Bcl-2, a combination of ILK and Bcl-2 or the recombination protein Cre as a control. After 5 days in our PEG capsule, viability analysis showed only 42(±9)% viability for the control cells, 95(±4)% viability for the ILK-overexpressing cells, 76(±4)% viability for Bcl-2 and 88(±6)% for the combination. All three experimental groups exhibited significantly higher viability than the control cells (*p* value <0.01). The ILK only and combination group (ILK plus Bcl-2) were statistically identical. These numbers are the average of three independent studies and representative images of the viability staining for the control and ILK groups are shown in figure 3. The images are projections of an 80 μm thick section of the gel obtained by confocal

microscopy. Uninfected cells displayed viability similar to that of the Cre-overexpressing cells indicating that the infection procedure and Cre overexpression did not reduce cell viability. The numerical values represent pooled data from six separate experiments.

Intact islets isolated from BALB/cByJ mice were infected to measure the impact of ILK or Bcl-2 overexpression on islet viability and function. Islets were infected with Cre, Bcl-2 or ILK and then encapsulated in a PEG gel. GSIS was monitored over 28 days with the results shown in figure 4. Insulin secretion from islets overexpressing Cre declined throughout the study with no insulin detected by day 28. Insulin secretion was higher for islets overexpressing Bcl-2 or ILK but dropped to about 50% of the initial value. ILK and Bcl-2 overexpression seemed to have a positive impact on insulin secretion, but the difference with the control samples was not statistically significant due to the variability between studies. The numerical values in figure 4 represent data from three (Bcl-2 and ILK) or four (Cre) separate experiments. Variability is expected for the GSIS procedure but was particularly high for ILK-overexpressing cells. This will be a topic of focus in future studies.

Viability analysis was performed for an additional set of genetically modified islets with an additional control of uninfected islets. Viability was measured by analysis of multiple images captured using confocal microscopy over a 28 day study. The results are shown in figure 5. Viability of each group correlated well with the GSIS results and the uninfected cells showed viability levels that were very similar to the islets infected with Cre. All four experimental groups showed viability greater than 95% 1 day after encapsulation but this value dropped on day 14 to 47.7, 51.4, 86.2 and 80.4% for uninfected, Cre-, ILK- and Bcl-2-overexpressing islets, respectively. The GSIS values tended to drop from the day 1 values more quickly than viability which would indicate that function was lost prior to cell death. The one exception to this was from the day 28 time point for cells overexpressing Bcl-2 or ILK. These islets showed 25.2% and 29.4% viability respectively on day 28. These values are greater than for uninfected or Cre-overexpressing islets (5.8% and 7.7%, respectively) as expected but lower than anticipated given the GSIS results for these groups on day 28 of those studies. Future studies carried out to a later end point would indicate whether that becomes a trend. Statistical analysis of the ILK-overexpressing cells showed a significant increase ($p < 0.05$) compared to the Cre-overexpressing controls on days 7, 14, 21 and 28. Cells overexpressing Bcl-2 exhibited a similar increase on days 14, 21 and 28.

Analysis of encapsulated MIN6 cells by Weber *et al* (2006) has shown that large MIN6 aggregates can be cultured in PEG gels for 21 days without loss of viability. A number of other cell types including marrow stromal cells (Nuttelman *et al* 2004), chondrocytes (Bryant and Anseth 2001, 2002 and Rice and Anseth 2004), osteoblasts (Burdick and Anseth 2002) and valvular interstitial cells (Masters *et al* 2005) have been encapsulated with similar procedures with maintenance of viability. Our study of dispersed cells showed that cell death started to occur in the days following encapsulation and after 10 days, few viable cells remained. Based on the improvement in viability at day 5 by overexpression of Bcl-2 or particularly ILK, the loss of viability seems to be mediated by stress caused by the foreign microenvironment presented by the PEG capsule. Cell-cell interactions seem to play an important role as clusters of MIN6 cells survive much longer under identical conditions than dispersed cells do. Functional analysis of encapsulated islets indicated a beneficial effect of Bcl-2 or ILK on maintenance of GSIS and viability. Collectively, the data show that encapsulated islets or MIN6 cells benefit from strategies to alleviate anoikis but this is not the only stress present. The drop of insulin release levels to less than 50% of the original level after 4 weeks may have been due to hypoxic stress and loss of function for central cells in our islets.

The results are in agreement with related previous studies including the work using Bcl-2 overexpression (Thomas *et al* 1999, 2001). A direct comparison with those studies is complicated by our encapsulation of the islets, but the use of Bcl-2 overexpression with unencapsulated islets provided the motivation for its use in these studies with encapsulated islets. We anticipated a beneficial effect to serve as a comparison for ILK. The similarity of the impact of ILK and Bcl-2 overexpression hints at the possibility of overlapping in the mechanisms by which the protective effect is manifested within the cell. This will be a topic of future studies in addition to extending the duration of our analysis.

4. Conclusions

Encapsulated MIN6 cells show rapid loss of viability when dispersed within the gel matrix compared to encapsulation as cell clusters. Encapsulated islet tissue showed no detectable insulin release following glucose stimulation after 4 weeks in the gel. Overexpression of ILK increased viability of encapsulated MIN6 cells and extended the duration of viability and insulin secretion from encapsulated islets. Strategies to alleviate hypoxia by reducing the dimensions of encapsulated islets into smaller clusters of tissue may not produce the anticipated favorable effect on viability and function due to anoikis. While ILK overexpression is certainly not the only method to address this issue, these results show the importance of considering the relationship between these phenomena and that hypoxia and anoikis be considered collectively.

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Figure 1.

A schematic of the role of ILK in a cell's response to integrin engagement. This process is important for suppression of apoptosis and could be diminished in the synthetic environment of a polymeric capsule. ILK overexpression could help to keep pro-survival signals active in the absence of proper cell-cell and cell-ECM interactions.

(This figure is in colour only in the electronic version)

Figure 2. MIN6 viability following encapsulation as measured by live/dead staining. Each time point expresses the amount of time since the cells were encapsulated.

Figure 3. Projections of an 80 μm thick section of the capsule showing live (green) and dead (red) MIN6 cells 5 days after encapsulation. The cells were infected to overexpress Cre (A), Bcl-2 (B), IKL (C) or ILK and Bcl-2 (D).

Figure 4. Glucose-stimulated insulin secretion is shown for genetically modified Balb/c islets encapsulated in a PEG gel over 4 weeks. The curves show average values for Cre- (diamond), Bcl-2- (square) and ILK- (triangle) overexpressing cells with standard deviation values. Each data point represents 3 or 4 individual experiments.

Figure 5.

Viability of islets infected to overexpress Cre (diamond), ILK (triangle), Bcl-2 (cross) or left uninfected (square) is shown over 28 days following encapsulation. Each data point represents an average value with standard deviation of cells counted from multiple images captured with a confocal microscope. Statistical significance compared to the Cre control ($p < 0.05$) is shown for ILK only (*) or both ILK and Bcl-2 (**) when applicable.