

Cell-cell contact influences proliferative marker expression and apoptosis in MIN6 cells grown in islet-like structures

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Cell-cell contact and beta cell growth

Abstract

Cell to cell interactions play an important role in the development and maintenance of the beta cell phenotype. Here, we have investigated whether E-cadherin plays a role in regulating the growth of insulin-secreting MIN6 cells configured as three-dimensional islet-like clusters (pseudoislets). Pseudoislets form by cell aggregation rather than by proliferation from individual cells, and attain the size of primary mouse islets after approximately 7-days maintenance in culture. E-cadherin is known to mediate homotypic cell adhesion between beta cells and has also been implicated in a number of cellular processes, including proliferation, apoptosis and differentiation. E-cadherin and its associated intracellular elements, α - and β -catenin, were up-regulated in MIN6 pseudoislets. Pseudoislet formation was associated with an increased expression of cyclin-dependent kinase inhibitors and a concomitant down-regulation of Ki67, suggesting an overall reduction in cellular proliferation. However, measurements of 5-bromo-2'-deoxyuridine incorporation revealed that there were no differences in the rate of MIN6 cell proliferation whether they were configured as monolayers or as pseudoislets, which is likely to be a result of their being a transformed cell line. Cells within pseudoislets were not necrotic, but apoptosis appeared to be up-regulated in the islet-like structures. However, no differential expression of Fas and FasL was detected in monolayers and pseudoislets. These results suggest that cell to cell interactions within islet-like structures may initiate anti-proliferative and pro-apoptotic signals.

Keywords: Islets of Langerhans, cell adhesion, E-cadherin, proliferation, pseudoislets.

Introduction

It is well established that intercellular interactions within islets of Langerhans are important for the functional competence of the islet. Cell to cell interactions are important for the development and maintenance of the beta cell phenotype, with insulin gene expression being up-regulated in beta cell clusters (2), and reduced by blocking interactions through integrins (11). Intercellular interactions are also required for normal beta cell secretory function. Thus, the insulin secretory responses of dispersed islet cells are much less than the integrated responses of intact islets, and are improved by reaggregation (30, 46, 20, 5). At least part of this effect is due to homotypic beta cell to beta cell interactions since insulin-secreting MIN6 cells show significantly enhanced insulin release when they are configured as three-dimensional islet-like structures known as pseudoislets (21). Pancreatic beta cells express a number of cell adhesion molecules (47, 10, 14) including the Ca^{2+} -dependent epithelial cell adhesion molecule, E-cadherin (ECAD) which plays an important role in regulating islet cell aggregation and in maintaining primary islet architecture (47, 14, 57). In accordance with this, the beta cell to beta cell interactions underlying the pseudoislet structure is dependent on ECAD, since pseudoislet formation was completely blocked by antibodies against the extracellular domain of ECAD (21).

In addition to its role as a cell adhesion molecule, ECAD mediates outside-in signalling and can regulate a number of cellular processes including proliferation, apoptosis and differentiation (43). ECAD has been identified as both an invasion- and proliferation- suppressor (44, 53), and misregulated ECAD expression or function can alter the pattern of epithelial growth and differentiation (19). ECAD is a single transmembrane domain glycoprotein (51) and it transduces signals from outside the cell through intracellular interactions with a protein family collectively termed catenins. β -Catenin interacts directly with the COOH-terminal domain of ECAD and α -catenin then associates with β -catenin. α -Catenin is an essential link between the cadherin/ β -

catenin complex and the actin cytoskeleton and its expression is essential for the cadherin complex to mediate functional adhesion (28). β -Catenin also plays an important role in regulating cell growth and differentiation in invertebrate and vertebrate organisms (13, 31) through interactions with transcription factors of the lymphoid enhancer binding factor (LEF)-1/T cell factor (TCF) family (3) thereby forming a bipartite transcription factor in which the DNA-binding and transactivation functions are contributed by TCFs and β -catenin, respectively. C-Myc (24) and cyclin D1 (52, 56) have been identified as targets of the β -catenin-LEF-1/TCF signalling pathway in colon carcinoma cells. It has been suggested that the proportion of cytosolic to membrane-associated β -catenin levels plays a critical role in regulating cellular responses to extracellular signals for cell adhesion and cell proliferation and ECAD can modulate β -catenin signalling by titrating free cytoplasmic β -catenin to the plasma membrane (27).

We have previously reported that the configuration of monolayer MIN6 cells into pseudoislet structures is accompanied by an up-regulation of ECAD expression (21), suggesting a causal link between ECAD expression and the functional changes associated with the aggregation of the cells into islet-like structures. In the current study we have investigated whether the enhanced cell-cell contact and up-regulation of ECAD expression in MIN6 pseudoislets plays a role in regulating the growth of MIN6 cells within the pseudoislets.

Materials and methods

MIN6 cells were kindly provided by Dr Y. Oka and Professor J.-I. Miyazaki (University of Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin/streptomycin, gelatin (from bovine skin), phosphate-buffered saline (PBS), trypsin-EDTA, 0.02% EDTA (0.5mM), were from Sigma-Aldrich (Poole, Dorset, U.K.). Foetal bovine serum, polyacrylamide gels (10%), molecular weight markers, sample buffer and PAGE buffers were from Invitrogen (Paisley, U.K.). Mouse monoclonal IgG2a E-cadherin antibody (clone 36) (0.25µg/ml) and mouse monoclonal IgG1 β-catenin antibody (0.2µg/ml) were from BD Biosciences Pharmingen, CA, U.S.A. Mouse monoclonal IgG1 α-catenin antibody (1µg/ml), mouse monoclonal IgG_{2b} p21 antibody (0.2µg/ml), goat polyclonal Ki67 antibody (2µg/ml); rabbit polyclonal Fas antibody (0.4µg/ml) and rabbit polyclonal FasL antibody (0.2µg/ml) were from Santa Cruz Biotechnology, Inc., CA, U.S.A. Mouse monoclonal IgG_{1κ} p27 antibody (0.1µg/ml) was from Merck Biosciences Ltd., Nottingham, U.K; Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, rabbit anti-goat IgG and goat anti-rabbit IgG were from Pierce Biotechnology Inc., Rockford, IL, U.S.A. The Enhanced Chemiluminescence (ECL) detection system and Hyperfilm were from Amersham Pharmacia Biotech International plc. (Buckinghamshire, U.K.). The colorimetric cell proliferation ELISA assay (Boehringer Mannheim, Germany) was used in this study to quantify the cell proliferation occurring in MIN6 cells. The ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kit was used to measure 3'-OH terminal DNA strand breaks as an indicator of apoptosis, essentially according to the manufacturer's instructions (Oncor, Gaithersburg, MD, U.S.A).

Cell culture and pseudoislet formation

MIN6 beta cells (passage 35-45) were maintained at 37°C/5%CO₂ in DMEM supplemented with 10% foetal bovine serum, 2 mmol/l glutamine, and 100 U/ml penicillin with 0.1 mg/ml streptomycin. The medium was changed every 3-4 days, and the monolayers were passaged and used for experiments when approximately 70% confluent. MIN6 pseudoislets were generated by culturing MIN6 cells for seven days on tissue culture flasks which had been precoated with gelatin (1% wt/vol), as described previously (21). In some experiments, pseudoislets were formed from an initial starting population comprising 50% wild type MIN6 cells and 50% MIN6 cells that stably expressed β -galactosidase under a cytomegalovirus promoter. 7 day-old pseudoislets were fixed in 4% paraformaldehyde, incubated overnight in 30% sucrose, embedded in optimum cutting temperature (O.C.T.) compound and frozen in liquid nitrogen. Frozen sections were cut at 7 μ m and stained with haematoxylin and eosin (H&E) stain using Gill's haematoxylin solution (17) for histological examination. For comparative purposes, a mouse pancreas was removed, fixed at room temperature in 4% paraformaldehyde and processed for wax sections. Sections were cut at 5 μ m and stained with H&E stain, as for pseudoislets. To further assess the viability of cells within pseudoislets, Hoechst 33342 / Propidium iodide staining was used as described (29). For the 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining, the cells were fixed with 0.05% glutaraldehyde (15 min., room temperature) and X-gal stain (20mM K⁺ ferrocyanide; 20mM K⁺ ferricyanide; 2mM MgCl₂; 1mg/ml X-gal) was added to the cells to detect β -galactosidase activity.

Immunoblot analysis of protein expression

Monolayer cells were detached using 0.02% EDTA solution to avoid proteolytic degradation of cell surface proteins, whilst the non-adherent pseudoislets were harvested by pipetting. Cells and pseudoislets were pelleted by centrifugation (5 min., 1000g) and washed twice with PBS. Protein extracts were prepared by sonication of the final pellets in a lysis buffer containing 20mM Tris,

2mM EDTA, 0.5mM EGTA, 1mM phenylmethylsulphonyl fluoride (PMSF), 50µg/ml leupeptin, 0.1% (v/v) β-mercaptoethanol, pH7.4, and total protein content was measured by the Bradford assay (6). Equivalent amounts of total protein from each sample were separated by NuPAGE gel electrophoresis on a NuPAGE Bis-Tris (Bis [2-hydroxyethyl] imino-tris [hydroxymethyl] methane)-HCl buffered (pH6.4) 10% polyacrylamide gel, and the resolved proteins were electrotransferred onto polyvinylidene difluoride membranes. Membranes were blocked (3-4h.) with 5% non-fat dried milk in a Tris-buffered saline (TBS)/Tween solution (0.5M Tris Base and 1.5M NaCl, pH 7.4 with 0.2% Tween 20) and immunoprobed for 3 hours with appropriate primary antibodies (0.1-2µg/ml in TBS/Tween). After repeated washing with TBS/Tween, membranes were incubated with the appropriate HRP-conjugated secondary antibody (1:5000, 2h.), and antibody binding was visualised using the ECL detection system according to the manufacturer's instructions. When necessary, primary and secondary antibodies were removed from immunoblots by incubation (25 min., 50°C) in a stripping buffer (0.7% β-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate (SDS), and 62.5mM Tris-HCl, pH 7.6) before reimunoprobing with alternative antibodies.

Cell proliferation and apoptosis in monolayer and pseudoislet MIN6 cells

DNA synthesis as a marker of cell proliferation was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) using a commercially available kit, essentially according to the manufacturer's instructions. Briefly, MIN6 cells grown as monolayers or as pseudoislets were incubated (37°C, 30-240 min.) in culture medium supplemented with 10µM BrdU labelling reagent. Pseudoislets were dispersed into single cell suspensions and the monolayer cells were detached from the tissue culture plastic substrate using 0.02% EDTA. Both cell populations were seeded into 96-well microtitre plates at a density of 10,000 cells per well, fixed and denatured, and incubated with an HRP-conjugated anti-BrdU antibody for 90 min. Antibody binding was visualised by incubation (20 min.) with an HRP substrate (tetramethylbenzidine). The reaction

was terminated by the addition of 1M H₂SO₄, and the coloured product was assessed by measuring the absorbance at 450 nm.

For measurements of apoptosis, detached monolayer cells and dispersed pseudoislet cells were fixed in 10% neutral-buffered formalin (10 min., 20°C) and dried onto microscope slides. The frequency of apoptosis was assessed by measuring 3'-OH terminal DNA strand breaks using the ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kit, essentially according to the manufacturer's protocol. The cells were viewed using a broad band fluorescence filter set, excitation wavelength 494nm, emission wavelength 523nm.

Statistical Analysis

Results are expressed as means \pm SEM. Differences between treatment groups were assessed by one way analysis of variance (ANOVA) and Student's T test or Bonferroni's multiple comparison test, as appropriate, and considered significant where $P < 0.05$.

Results

Formation of MIN6 pseudoislets

Figure 1 (A-C) shows light micrographs of a primary mouse islet of Langerhans (Fig.1A) and of MIN6 cells configured as pseudoislets by maintenance in culture on a gelatin substrate (Fig.1B, C). After seven days in culture (Fig. 1B) the MIN6 pseudoislets were of similar size and appearance to the primary mouse islets, and they did not further increase in size with prolonged culture up to 22 days, although there was some evidence of monolayer cell outgrowth from pseudoislets with this prolonged culture (Fig. 1C). Histological examination of H&E stained sections (Fig. 1D, E) revealed that pseudoislets had a similar morphology to primary mouse islets *in situ*, with no evidence of marked necrosis in the centre of the pseudoislets, irrespective of the size of the pseudoislet. In addition, Hoechst /propidium iodide staining of pseudoislet cells confirmed that there was no extensive necrosis in the core of the pseudoislets (data not shown). Pseudoislets formed from a mixture of wild type and β -galactosidase expressing MIN6 cells (50%/50%) contained an approximately equal number of each type of cell distributed throughout the pseudoislet as assessed by X-gal staining (Fig. 1F), consistent with pseudoislets forming by cell aggregation rather than by proliferation from a single cell, or small group of cells.

Expression of ECAD and catenins in monolayer and pseudoislet MIN6 cells

Immunoblot measurements of ECAD expression during pseudoislet formation demonstrated increased ECAD expression, as shown in Figure 2. In parallel with the increased ECAD content of pseudoislets, we also observed increases in intracellular proteins that interact with the cytosolic domain of ECAD. Immunoblot analysis of protein extracts from pseudoislets and from equivalent MIN6 cells grown as monolayers revealed increased levels of both α -catenin and β -catenin in pseudoislet extracts compared to protein-matched monolayer extracts, as shown in Figure 3. Densitometric analysis revealed an approximately 50% increase in the expression of ECAD, α -

catenin and β -catenin in MIN6 cells configured as pseudoislets, suggesting a parallel and coordinated up-regulation of the interacting proteins. β -Catenin levels and cellular location can be modified by signals acting through the Wnt/Frizzled extracellular signal recognition pathway (18). However, we were unable to detect either the receptor Frizzled or its agonist Wnt in MIN6 cells, whether configured as monolayers or as pseudoislets, although Frizzled immunoreactivity was detected in extracts of mouse heart (data not shown). These results suggest that this signal transduction pathway is not involved in the regulation of MIN6 cell proliferation or differentiation.

Proliferation and apoptosis in monolayer and pseudoislet MIN6 cells

To investigate whether pseudoislet formation was associated with changes in cell proliferation the expression of the proliferation-associated antigen, Ki67, and of the cyclin-dependent kinase inhibitors (CKIs) p21 and p27 were measured. The immunoblot in Figure 4 (upper panel) shows that pseudoislets contained less Ki67 than did equivalent monolayer cells, consistent with reduced proliferation in cells configured as pseudoislets. Measurements of p21 and p27 immunoreactivities in the same extracts demonstrated increased levels in pseudoislets (Fig. 4, middle and lower panels, respectively), again consistent with reduced proliferation since p21 induces cell cycle arrest by binding to and inhibiting the Proliferating Cell Nuclear Antigen (PCNA), cyclinE-cyclin-dependent kinase (cdk)2 and cyclinD-cdk4/6 complexes, while p27 acts by inhibiting the activity of the latter two complexes. Densitometric measurements revealed an approximate 40% decrease or increase in Ki67 and CKI expression, respectively, upon pseudoislet formation.

However, in contrast to the proliferative markers, direct measurements of DNA synthesis by BrdU incorporation clearly demonstrated that there were no differences in the rate of proliferation of MIN6 cells whether they were configured as monolayers or as pseudoislets. Figure 5 shows the

increased incorporation of BrdU into MIN6 cells with time, and demonstrates that the rate of BrdU incorporation was not significantly different between cells configured as monolayers or as pseudoislets at any time point ($P > 0.2$ at all time points measured). These data indicate that the rate of DNA synthesis, and thus cell proliferation, was not influenced by the enhanced cell to cell contact in MIN6 pseudoislets over the time course of pseudoislet formation used in the present studies.

Estimation of the degree of apoptosis in MIN6 cell population by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay assessment of DNA damage demonstrated that there was a much higher frequency of apoptotic nuclei in cells configured as pseudoislets than in equivalent cells maintained as monolayers, as shown in Figure 6. Receptor-induced apoptosis mediated by an interaction between Fas and its ligand FasL, is a major inducer of apoptosis in pancreatic β -cells (9, 35). Immunoblot analysis for Fas and FasL demonstrated that both proteins were expressed in MIN6 cells maintained as monolayers or as pseudoislets, as shown in Figure 7. However, in contrast to ECAD and the catenins (Figs. 2 and 3) the expression of Fas and FasL was not influenced by configuring the cells as pseudoislets (Fig. 7).

Discussion

Primary islets of Langerhans dispersed into cell suspensions can spontaneously reaggregate *in vitro* into anatomically correct islet-like structures (20) and regain some of the secretory responsiveness that is lost on islet dispersal (30, 46, 5). The precise nature of the intercellular signalling involved in maintaining integrated islet function is uncertain, although there is experimental evidence for the involvement of direct communication via gap junctions (8) and for paracrine effects of adenine nucleotides (15, 23), nitric oxide (41, 42, 48), carbon monoxide (26), glucagon (46) and insulin (1), among others. We have previously demonstrated the ECAD-dependent formation of islet-like structures (pseudoislets) from a homogenous beta cell population (21), and a concomitant enhancement of secretory function (21, 22, 45), suggesting that homotypic beta cell interactions are largely responsible for the gain of function in intact islets. The current study has shown that MIN6 pseudoislets attain a similar size and morphology to primary mouse islets within seven days of culture and then remain static in size for over 20-days culture *in vitro*. MIN6 cells are a transformed beta cell line which expresses the Simian virus 40 (SV40) large T antigen (TAG) and they proliferate when maintained in monolayer culture on tissue culture plastic (38). By tracing the origin of the cells within individual pseudoislets using MIN6 cells that stably expressed β -galactosidase we have demonstrated that the proportion of lacZ-expressing cells within pseudoislets reflected that of the original cell suspension, indicative of pseudoislet formation by coalition of cells rather than by proliferation from individual founder cells. Previous studies analysing the growth of tumour spheroids have reported the phenomenon of growth saturation, whereby the spheroid growth rate decreases with increasing diameter, until a stable maximal size is attained (4). These spheroids generally comprise a viable cell rim approximately 100 μ m in diameter surrounding a core of apoptotic and necrotic cells that is attributed to microenvironmental stress caused by increasing diffusion gradients and to breakdown products of necrotic cells (16, 4). However, this effect was only observed in spheroids which had attained

diameters of approximately 4000 μ m, so it is unlikely to be responsible for the size limitation of pseudoislets which have a diameter (~100 μ m) equivalent to the viable rim surrounding the necrotic core of the spheroid. Our histological analysis of pseudoislets could find no evidence of necrosis at the core over the time course used in our studies, suggesting nutrient access by diffusion was adequate to maintain the cells within the pseudoislet. The static size of the pseudoislets therefore implies an inherent ability of the MIN6 cells to regulate pseudoislet size by modulating either cellular proliferation or apoptosis, or both.

Cell to cell contact through cell adhesion molecules is known to influence proliferation in a number of cell types (44, 53, 43). Our earlier studies demonstrated that pseudoislet formation was dependent on the expression of ECAD (21) and we have now shown that pseudoislet formation is associated with increased levels of ECAD and the associated intracellular elements, α - and β -catenin. These observations are consistent with homotypic ECAD-mediated interactions between pseudoislet cells being involved in both cell adhesion and in inwardly-directed catenin-mediated signalling to regulate cell proliferation. In accordance with this, immunoblotting revealed that pseudoislet formation was associated with increased expression of the CKIs, p21^{Cip1} and p27^{Kip1}, and reduced expression of Ki67, suggesting that pseudoislet size was regulated by decreased cellular proliferation as a result of enhanced cell to cell contact. Although the changes in protein expression were relatively small they were reproducible and could not be attributed to differences in protein loading because total protein content in the samples was quantified before loading, and the increased expression of ECAD, catenins and CKIs were detected in the same samples showing decreased expression of Ki67.

However, in contrast to the measurements of protein expression, direct measurements of DNA synthesis by assessing BrdU incorporation showed no differences between cells configured as

monolayers or as pseudoislets, clearly demonstrating that the rate of MIN6 cell proliferation was not affected by 7 days cell-cell contact within a pseudoislet structure. The lack of effect of anatomical configuration on the proliferative capacity of MIN6 cells is perhaps not unexpected given the nature of their transformation. During the G1 phase of the cell cycle the cell integrates mitogenic and growth inhibitory signals and makes the decision to proceed, pause or exit the cell cycle. The constitutive expression of SV40 TAg in MIN6 cells overrides the normal control mechanisms that pause cells in the G1 phase by binding to and inactivating the retinoblastoma (Rb) and p53 tumour suppressor proteins. Progression through G1 phase is controlled by the G1 phase cyclin-dependent kinases (Cdks) Cdk4 (or Cdk6) and Cdk2, the activities of which are controlled by CKIs, including p21^{Cip1} and p27^{Kip1} (32). The abundance of CKIs is tightly regulated and our observations of their increased expression in the presence of continued DNA synthesis and proliferation in pseudoislets is consistent with an inward signal to reduce proliferation being overridden by the actions of SV40 TAg. This conclusion has implications beyond the scope of the present study. Thus, our results show that changes in the expression of the commonly used proliferative markers Ki67 and CKIs do not unambiguously reflect changes in the rate of proliferation, and should always be supported by more direct measurements of mitosis such as DNA synthesis. Secondly, many studies of the regulation of beta cell proliferation have used transformed cell lines to avoid the problems inherent in obtaining and using primary islets of Langerhans (55, 40, 37). The current results suggest that these may be inappropriate experimental models for such studies.

Pancreatic beta cells are responsive to both mitogenic and apoptotic stimuli, and the maintenance of an appropriate beta cell mass is a balance between the rate of proliferation and the rate of apoptosis (49, 33). Our measurements of apoptosis in MIN6 cell populations using two different experimental end-points demonstrate that an increased rate of apoptosis detected in cells configured as pseudoislets for 7 days is the primary determinant of pseudoislet growth. The

incidence of apoptosis was considerably greater in cells within MIN6 pseudoislets than in equivalent monolayer cells, as shown by the much higher occurrence of apoptotic nuclei in pseudoislet cells and by the increased DNA fragmentation detected in pseudoislet extracts. The parallel characteristics of an increased rate of apoptosis with no change in proliferation might suggest a dissociation between the two processes in MIN6 cells configured as pseudoislets. A variety of intrinsic and extrinsic stimuli are reported to induce apoptosis in beta cells (33, 36) but one mechanism of direct relevance to their anatomical configuration is the cell death surface receptor, Fas, and its ligand, FasL. Interactions between Fas and FasL induce apoptosis in Fas expressing cells through a pathway leading to the activation of caspase 8, and the subsequent activation of the downstream effector caspases 3, 6 or 7 that are responsible for cellular disassembly (12, 39). Human primary beta cells constitutively express FasL, and the induction of Fas expression in response to environmental stimuli can lead to Fas/FasL-dependent apoptosis (54, 34, 35). In accordance with this, our immunoblotting experiments confirm that both Fas and FasL were expressed in MIN6 cell populations configured either as monolayers and pseudoislets, although these measurements could not determine whether individual cells expressed both molecules. Since the formation of three-dimensional pseudoislets is accompanied by greatly increased contact between cells it seems inevitable that this will enhance the likelihood of Fas/FasL interactions, and thus of an increased frequency of apoptosis in cells configured as pseudoislets. This increased rate of apoptosis is the most likely mechanism through which pseudoislets formed from proliferative MIN6 cells maintain a limited size. In contrast to other endocrine organs, the average size of islets of Langerhans is fairly consistent across species of very different sizes (25, 7), with the increased beta cell mass being achieved by an increase in the number of islets in the pancreas. We propose that the limitation on islet size relates to the necessity to integrate function between the individual cells within the islet, and that the mechanisms that limit islet size are an inherent property of beta cells.

In conclusion, MIN6 cells spontaneously form islet-like structures *in vitro* through an ECAD-dependent process. Cell to cell interactions within these structures initiate anti-proliferative signals but do not reduce cell proliferation, presumably because they are overridden by the actions of the SV40 Tag. In contrast the cell-cell interactions increased the rate of apoptosis, limiting pseudoislet size to that of primary islets of Langerhans. Experimental manipulation of these cell to cell interactions may provide novel therapeutic targets through which to maintain a functional beta cell mass and prevent secondary beta cell failure in type 2 diabetes or after islet transplantation therapy.

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Figure Legends

Figure 1: Formation of pseudoislets from MIN6 cells.

A-C. Morphology of mouse islets and MIN6 pseudoislets. The photomicrographs show mouse islets (A) or MIN6 pseudoislets maintained in culture for 7 days (B) or 22 days (C). Bars show 100 μ m for A and B; 10 μ m for C.

D-E. Histological sections of a mouse islet and a MIN6 pseudoislet. H&E staining of a primary mouse islet in a section of pancreas (D) and of a MIN6 pseudoislet (E) showed similar structures with no evidence of necrosis. Bars show 20 μ m.

F. β -Galactosidase expression in chimeric pseudoislets. β -Galactosidase expression was assessed in pseudoislets formed from a 50%/50% mixture of wild type MIN6 cells and MIN6 cells expressing β -galactosidase. The distribution of cells expressing β -galactosidase, assessed by the blue staining, was consistent with the formation of pseudoislets from a random aggregation of cells. Bars show 50 μ m.

Figure 2: ECAD expression in monolayer and pseudoislet MIN6 cells.

An anti-ECAD antibody detected a protein in monolayer and pseudoislet cell extract samples with an apparent molecular weight of 120kDa, corresponding to the molecular weight of ECAD. ECAD expression was increased in pseudoislet samples compared to their protein-matched monolayer counterparts. Extracts of monolayers (M) or pseudoislets (PI) were loaded at 10 μ g or 20 μ g, as shown. Results are representative of three similar experiments.

Figure 3: Catenin expression in monolayer and pseudoislet MIN6 cells. Antibodies against β -catenin and α -catenin detected proteins with apparent molecular weights of 92kDa and 102kDa, corresponding to the molecular weight of β -catenin and α -catenin, respectively, in protein-matched MIN6 monolayer (M) and pseudoislet (PI) samples. The expression of both catenins was higher in MIN6 pseudoislets compared to their protein-matched monolayer counterparts. Results are representative of three similar experiments.

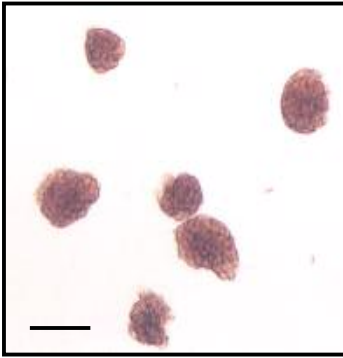
Figure 4: Expression of proliferation markers in monolayer and pseudoislet MIN6 cells. The upper panel demonstrates the presence of an immunoreactive band with an apparent molecular weight of 67kDa, corresponding to the molecular weight of the proliferation-associated nuclear antigen, Ki67, in protein-matched monolayers (M) and pseudoislets (PI); the lower panels show immunoreactive bands of 21kDa and 27kDa, corresponding to the molecular weights of the CKIs, p21^{Cip1} and p27^{Kip1}. Results are representative of three similar experiments.

Figure 5: Rate of BrdU incorporation into MIN6 cells configured as monolayers or pseudoislets. MIN6 monolayers and pseudoislets were exposed to growth medium containing 10 μ M BrdU labelling reagent for 30, 60, 120 or 240 min. Pseudoislets were then dispersed and monolayers were detached from the flask, both cell populations were counted and seeded out at densities of 10,000 cells per well. The amount of incorporated BrdU was determined by measuring the absorbance at 450nm. No significant difference in the rate of BrdU incorporation into MIN6 beta cell DNA within monolayers and pseudoislets was detected at any of the time points measured (points are mean \pm SEM, n=9, P>0.2).

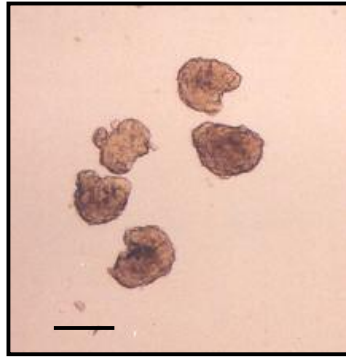
Figure 6: Detection of apoptosis in MIN6 cells configured as monolayers and pseudoislets by fluorescence microscopy. MIN6 monolayers and pseudoislets stained with the ApopTag Plus Fluorescein Kit were viewed and counted. MIN6 monolayer cells and pseudoislets were cultured for seven days in the presence of growth medium containing 5.5mM glucose. A significantly greater proportion of pseudoislet cells contained apoptotic nuclei, compared to equivalent monolayer cells (bars show means \pm SEM, n=6, *, P<0.01).

Figure 7: Fas and FasL expression in MIN6 monolayers and pseudoislets. Cell extracts of monolayers and pseudoislets were resolved on a 10% polyacrylamide gel and Fas protein (48kDa) or FasL protein (38kDa), upper and lower panels respectively, were detected by Western blotting. Lanes 1 and 3 contain 20 and 10 μ g protein, respectively, from cell extracts of monolayers. Lanes 2 and 4 contain 20 and 10 μ g protein, respectively, from cell extracts of pseudoislets. Results are representative of three similar experiments.

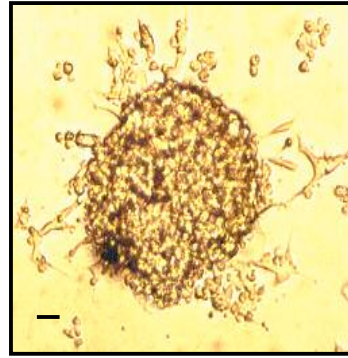
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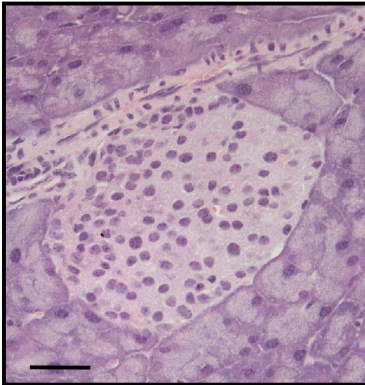
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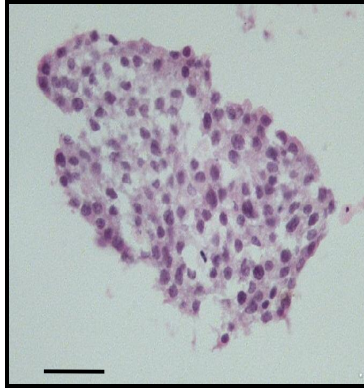
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D



E



F

