Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet–matrix relationship

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Abstract

Islet transplantation is associated with a high rate of early graft failure, a problem that remains poorly understood. It is probable that the destruction of the islet microenvironment and loss of tropic support that occur during isolation lead to compromised survival. The purpose of this study was to determine the role of matrix–integrin interactions on beta-cell survival and function following islet isolation. Canine islets were obtained by conventional methods. Immediately after isolation, the peri-insular basement membrane (BM) was absent. The ability of islets maintained in suspension culture to attach to a collagen matrix declined progressively over 6 days. Attachment could be blocked by an arginine-glycine-aspartate (RGD) motif-presenting synthetic peptide, thereby implicating an integrin-mediated process. Characterization of cell surface integrins by immunocytochemistry (ICC) demonstrated that the expression of integrins α3, α5 and αV diminished during the culture period. This change was coincident with both a decrease in beta-cell function (proinsulin gene expression, islet insulin content and stimulated insulin release) and a rise in beta-cell death from apoptosis, as determined by *in situ* cell death detection (TUNEL) assay. These adverse events were prevented or delayed by exposure of islets to matrix proteins. In conclusion, routine islet isolation disrupts the cell–matrix relationship leading to a variety of structural and functional abnormalities, including apoptotic cell death. These alterations can be diminished by restoration of a culture microenvironment that includes matrix proteins.

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Introduction

Islet transplantation has the potential to become a widely applicable treatment for insulin-dependent diabetes. Unfortunately, islet transplantation is associated with a high rate of primary non-function, a problem which remains poorly understood. In contrast, whole pancreas transplantation is capable of permanently reversing the hyperglycemic state and may prevent or delay at least some secondary complications of the disease (Orloff *et al.* 1987, 1988, Hering *et al.* 1994). This dichotomy suggests that there is a fundamental biologic difference between vascularized pancreas and purified islet cell grafts. It is probable that the destruction of the islet microenvironment and the loss of tropic support that occur during isolation, purification and in the pre-transplant culture period, subject the islets to a cellular stress that could impair beta-cell function and survival (Bissell *et al.* 1982, Rosenberg & Duguid 1996).

Extracellular matrix (ECM) is arguably the most important component of the islet microenvironment. It is a dynamic complex of different molecules that serves as a cellular scaffold, and functions in addition, to regulate both differentiation and survival. ECM is found in two forms – interstitial matrix and basement membrane (BM). Islet BM is composed of collagen IV, laminin and fibronectin (FN) (van Deijnen *et al.* 1993). As enzymatic digestion during islet isolation is poorly controlled, not only is the interstitial matrix destroyed, but the peri-insular BM may be readily digested as well (Wang *et al.* 1999). Disruption of the cell–matrix relationship (Frisch & Francis 1994, Bates *et al.* 1995, Schwartz & Bennett 1995) and the loss of BM both lead to the induction of apoptosis (Raff 1992, Meredith & Schwartz 1997). Recent evidence implicates integrins in this process (Juliano & Haskill 1993, Schwartz & Ingber 1994, Meredith *et al.* 1996, Meredith & Schwartz 1997, Wang *et al.* 1999).

The integrins are a diverse class of αβ heterodimeric receptors through which cells interact with matrix proteins, such as FN and collagen, providing the physical basis for cell adhesion (Hynes 1992, Juliano & Haskill 1993, Schwartz & Ingber 1994, Meredith *et al.* 1996). More importantly, though, integrins transduce biochemical signals both into and out of cells (Schwartz *et al.* 1995). The cytoplasmic domains of integrins interact with several structural and signaling proteins and consequently participate in the regulation of cell shape, motility, growth and differentiation (Dedhar & Hannigan 1996). It may be

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relevant that FN acts through the α5β1 integrin to protect cells from apoptosis (Zhang *et al.* 1995).

The question arises whether beta-cell function and survival prior to transplantation can be enhanced by trying to re-establish the cell–matrix relationship. To examine this issue, we isolated canine islets to study the role of the matrix–integrin interaction on islet beta-cell function and survival in culture.

Materials and Methods

Islet isolation and purification

For islet isolation, pancreata from four mongrel dogs of both sexes (2–4 years old with body weight 20–25 kg) were resected under general anesthesia in accordance with Canadian Council for Animal Care (CCAC) guidelines. The pancreatic ducts were cannulated prior to removal, and the glands were then rapidly excised. Islets were isolated from the surrounding exocrine tissue by enzymatic digestion using an HBSS solution (Gibco, Burlington, ON, Canada) containing DNase I (0·1 mg/ml) and Liberase CI (1·25 mg/ml) (Boehringer Mannheim, Indianapolis, IN, USA) at 37 °C according to established protocols (Horaguchi & Merrell 1981, Ricordi 1992). Purification was achieved by density gradient separation in a three-step EuroFicoll gradient using a COBE 2991 Cell Processor (COBE BCT, Denver, CO, USA) (Ricordi 1992). The final preparation consisted of 90% dithizone-positive particles with diameters ranging from 50 to 500 μm.

Experimental design

After overnight recovery in CMRL1066 supplemented with 10% FBS (GIBCO), three experimental groups were established: Group I-control (Ctrl) islets (1000 islets per dish, 60 × 15 mm plastic Petri dish, Fisher, Montréal, Québec, Canada) placed in suspension culture in CMRL1066 supplemented with 10% FBS; Group II-islets (1000 islets per dish) maintained as in Group I, but supplemented with soluble FN 10 μg/ml (Peninsula Laboratories, Belmont, CA, USA); and Group III-islets (1000 islets per flask, 25 cm^2 Corning flask, Fisher) grown on rat tail collagen-coated plastic (Coll), in the same medium as Group I. Islets were cultured in 95% air/5% CO_2 at 37 °C and the medium was changed on alternate days.

Representative islets from each group were examined after overnight recovery (day 0), and then on days of 1, 2, 3, 4 and 6 using the following investigations.

Studies of islet structure, function and survival

Islet morphology Islets were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C and embedded in 2% agarose following a standard protocol of dehydration and paraffin embedding. A set of ten serial sections (thickness 3 μm) was cut from each paraffin block.

Consecutive sections were processed for routine histology and reticulin staining (Wang *et al.* 1999), and immunostained for pancreatic hormones (insulin and glucagon, Biogenex, San Ramon, CA, USA), matrix proteins (collagen IV and Laminin, Sigma, St Louis, MO, USA) and integrins α3, α5 and αV (Chemicon International, Temecula, CA, USA), using the AB complex method (streptavidin–biotin horseradish peroxidase complex; Dako, Glostrup, Denmark), as described previously (Wang *et al.* 1994). For integrin α3 and collagen IV, sections were pretreated with 0·1% trypsin. The sections were incubated overnight at 4 °C with the appropriate primary antibodies. Bound antibodies were visualized by using biotinylated goat anti-mouse and goat anti-rabbit immunoglobulin G (IgG) (Amersham International plc, Amersham, Bucks, UK), and developed with DAB (3,3′-diaminobenzidinetetrahydrochloride; Sigma) as chromogens. Negative controls involved the omission of the primary antibodies.

For detection of proinsulin mRNA, the *in situ* hybridization (ISH) kit for human proinsulin mRNA was obtained from Novocastra (Burlington, ON, Canada). A fluorescein labeled oligonucleotide cocktail was applied to consecutive sections (3 μm) of islets from day 0 and day 3 that were mounted on sialinized slides (Shorrock *et al.* 1991). Pretreatment consisted of proteinase K at 10 μg/ml in proteinase K buffer for 30 min at 37 °C. The sections were hybridized for 2 h at 37 °C with 20 μl probe hybridization solution. Post-hybridization washes took place in TBS containing 0·1% Triton X-100. Slides were then incubated with rabbit Fab anti-fluoroscein isothiocyanate conjugated to alkaline phosphatase antibody (diluted 1:200) for 30 min at room temperature. The reaction product was visualized by an enzyme-catalysed color reaction using a nitro blue tetrazolium and 5′-bromo-4-chloro-3-indolyl-phosphate kit. The specificity of proinsulin mRNA oligonucleotide probes was tested by omission of the probes and pretreatment of the sections with RNase (100 μl/ml in 2HSSC/10 mM $MgCl_2$ at 37 °C for 1 h), which abolished all reactivity (Wang *et al.* 1994).

Quantification of immunocytochemistry and *in situ* hybridization Computer-assisted measurements of total integrin α5-, insulin-, proinsulin- and glucagon-immunoreactive areas in the sections from each experimental group was carried out at the previously indicated time points during the post isolation period. Of the integrins examined by immunocytochemistry (ICC), we chose to quantify only the extent of α5 staining because of its known association with apoptosis (Zhang *et al.* 1995).

Islet cross-sectional areas were traced manually with a mouse (Wang *et al.* 1994) using an Olympus BX60

microscope connected by a video camera to a Compaq computer equipped with Image-Pro Plus software version 4·0. Three randomly selected sections of the first ten islets from each group were measured. This procedure was performed for each of the four different islet isolation and total 120 islets were examined per group. The ratio of the integrin α5-, insulin-, proinsulin- and glucagon-immunoreactive area to the whole islet area was determined and expressed as the percentage of cross-sectional area stained.

Adhesion assay An adhesion assay is a common method used to delineate normal cell–matrix interactions and to assess the functional role of integrins (Weinel *et al.* 1992). We have adapted this technique and developed an islet adhesion assay to determine whether islets bind specifically to dishes coated with the ECM protein in collagen I.

Six-well tissue culture dishes were coated with rat tail collagen by applying neutralized collagen onto the surface of each dish. After allowing 10 min for gel formation, the coated dishes were incubated with a BSA blocking solution containing 1% BSA, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ in PBS pH 7·4 for 1 h, to block nonspecific binding sites, and then washed extensively in PBS. Non-coated dishes were blocked with a BSA blocking solution.

Islets from each group were harvested at the specified time points and resuspended in CMRL1066 into coated and non-coated wells (100 islets/well) and incubated for 1 h at 37 °C. At the end of the incubation period, unattached islets were washed off by repeated rinses in PBS. Attached islets were stained with dithizone and counted using a conventional light microscope. The number of islets attached to coated and non-coated wells was counted, and the data are reported as the percentage of islets attached. For each experiment, values are the average for three dishes and each experiment was repeated four times (i.e., four isolations were performed).

To confirm that the observed adhesion was integrin-mediated, the effect of a synthetic integrin-binding peptide on adhesion was determined. After overnight recovery, islets were pre-incubated at 37 °C for 30 min in the presence of the RGD motif-containing GRGDSP peptide or the control peptide GRGESP (Peninsula Laboratories) at doses ranging from 100 to 400 μg/ml, and then the adhesion assay performed as above.

Islet insulin content, basal insulin release and islet response to glucose challenge The islet insulin content and basal 24 h insulin release from each experimental group at the specified time points were measured by using a solid-phase radioimmunoassay (Immunocorp, Montréal, Québec, Canada) with a sensitivity of 26·7 pmol/l (0·15 ng/ml), an interassay variability of <5%, and an accuracy of 100%. The kit uses anti-human antibodies which cross-reacts with canine insulin, as well as porcine and bovine insulin. At the same time, the DNA content of the samples was determined. Basal insulin release was expressed as ng per 10^3 islets, and insulin content was shown as μg per μg DNA (Yuan *et al.* 1996).

Islets were subjected to an acute glucose challenge on day 0 and day 2. Cultured islets from each experimental group (100 per group in duplicate) were washed in RPMI-1640 (without glucose, Sigma) supplemented with 0·5% BSA and incubated in RPMI 1640 containing 2·2 mM glucose for two consecutive periods of 60 min. This was followed by two consecutive 30-min exposures to 22 mM glucose and 22 mM glucose supplemented with 50 μM IBMX respectively. Finally, islets were washed in RPMI 1640 and incubated for 1 h at a glucose concentration of 2·2 mM. Samples for insulin analysis were collected at the end of each incubation period. A static glucose stimulation index was calculated by dividing the insulin output from the high glucose plus IBMX incubation by the insulin output during low glucose incubation.

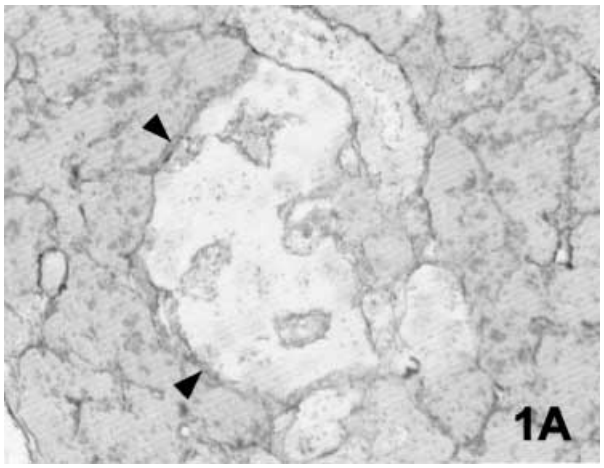
***In situ* cell death detection (TUNEL)** To identify apoptotic cells, a TUNEL (TdT-mediated dUTP-X Nick End Labeling) assay (Kaiser *et al.* 1995) was carried out at the specified time points on consecutive sections cut from the paraffin blocks. The TUNEL kit from Boehringer Mannheim was used. After deparaffinization, the sections were pretreated with 0·1% trypsin, and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP at 3′-end for 60 min at 37 °C. Islet cells containing damaged DNA were marked by an anti-fluorescein antibody conjugated with horseradish peroxidase and developed a dark brown color in the nucleus by DAB substrate reaction. The negative controls either lacked the terminal deoxynucleotidyl transferase, or omitted anti-FITC antibody.

To calculate an apoptotic cell index, the number of cells positive for the TUNEL reaction was determined and expressed as a percentage of the total number of cells counted. For each experimental group and time point, at least 500 islet cells were counted per section.

To identify specific cell types undergoing apoptosis, a double labeling reaction was used. The same sections which had been reacted with the TUNEL reagent were subsequently stained with mouse monoclonal anti-insulin or rabbit anti-glucagon antibodies to demonstrate pancreatic hormones. These antibodies were detected using the ABC/AP method (streptavidin–biotin–alkaline phosphatase complex) and NAMP/fast red (naphthol-AS-MX-phosphate/fast red TR salt, Sigma) as chromogen to obtain a red reaction in the cytoplasm.

Statistical analysis

Data obtained from the four different islet isolations are expressed as means ± S.E.M. The difference between



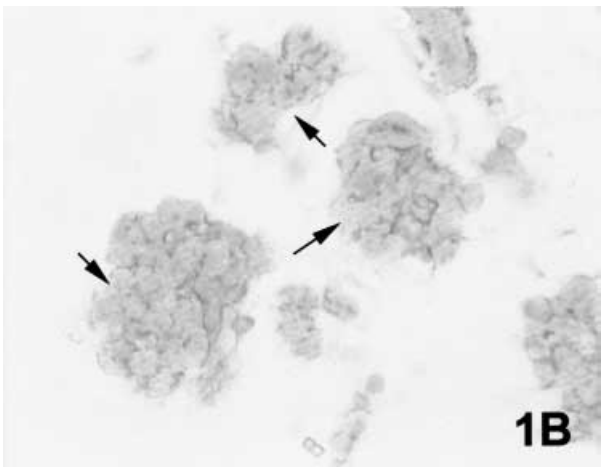


Figure 1 Reticulin staining in canine pancreas (A) and isolated islets on day 0 (B). An intact BM is demonstrated around the islet in the pancreas (arrowheads), but it is absent immediately after isolation (arrows). × 400

groups was evaluated by one-way analysis of variance, paired and unpaired Student's *t*-test.

Results

Islet morphology

Following isolation, islet morphology was preserved, with the core region of primarily beta-cells surrounded by a mantle of non-beta cells. However, the continuous peri-insular BM was lost as a result of the enzymatic digestion (Fig. 1). Immunocytochemical localization of laminin and collagen IV demonstrated that they were components of the peri-insular BM in the pancreas, which were lost during isolation. After 3 days in culture, the islet core in both control and FN groups exhibited varying degrees of cell necrosis. Although this pattern of central necrosis was

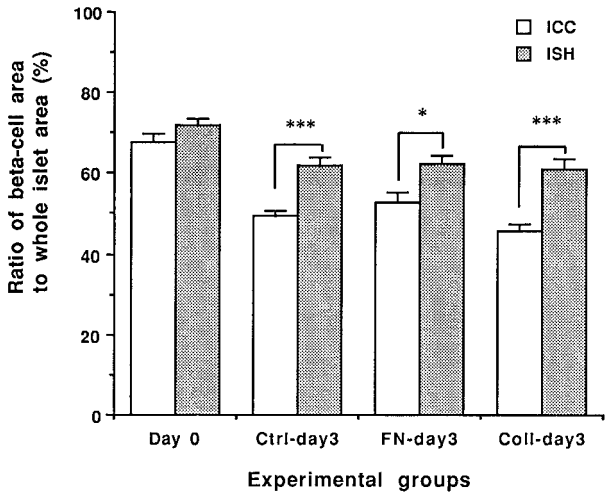


Figure 2 Comparison of beta-cell area based on ISH for proinsulin mRNA and immunostaining for insulin peptide (ICC) in isolated canine islets on day 0 and day 3. Significant differences are shown on day 3 of culture in all study groups. Coll, collagen. *P<0·05, ***P<0·001.

not observed in islets cultured on collagen, islets in this group lost their spherical shape as cells spread out onto the surface of the matrix after day 4.

To identify all beta cells regardless of the presence or absence of immunoreactive insulin, ISH for proinsulin mRNA was performed on day 0 and day 3. There was no significant difference between the size of the beta-cell area detected by ISH or ICC at day 0 (Fig. 2). On day 3, however, there was a divergence of results obtained using these two techniques. Immunocytochemical staining showed an apparent decrease in the area of beta cells in all groups, especially in the collagen group, in which there was a 25% reduction in the beta-cell area compared with ISH (P<0·001).

Morphometric analysis for insulin- and glucagon-immunoreactive areas was performed to get a more dynamic view of islet morphology over time. In the control and collagen groups, the percentage of the islet area that was immunoreactive for insulin decreased during the culture period, reaching 47 and 43% respectively of the initial values by day 6 (Fig. 3A). In the FN group, there was a slight increase in the insulin-immunoreactive area on the first day compared with day 0. By day 6, however, observations in this group were similar to the other two groups. The size of the glucagon-immunoreactive area showed no significant differences either between groups or over time (Fig. 3C).

Integrin expression and function in isolated canine islets

Integrins α3, α5 and αV were demonstrated by ICC to be present on islets after isolation. However, integrin

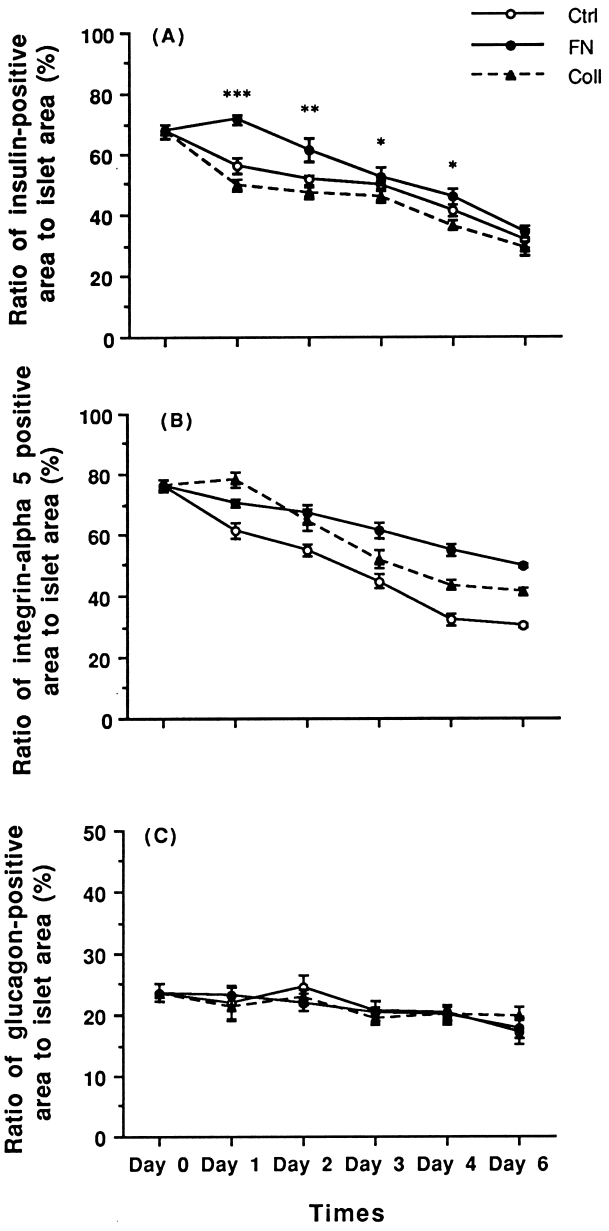


Figure 3 The ratio of insulin (A), integrin α5 (B) and glucagon (C) immunoreactive area as a percentage of islet area in isolated canine islets at different times in culture. Significant differences in integrin α5-immunoreactive area are shown between the control and both the FN and collagen (Coll) groups at all times. *P<0·05, **P<0·01, ***P<0·001.

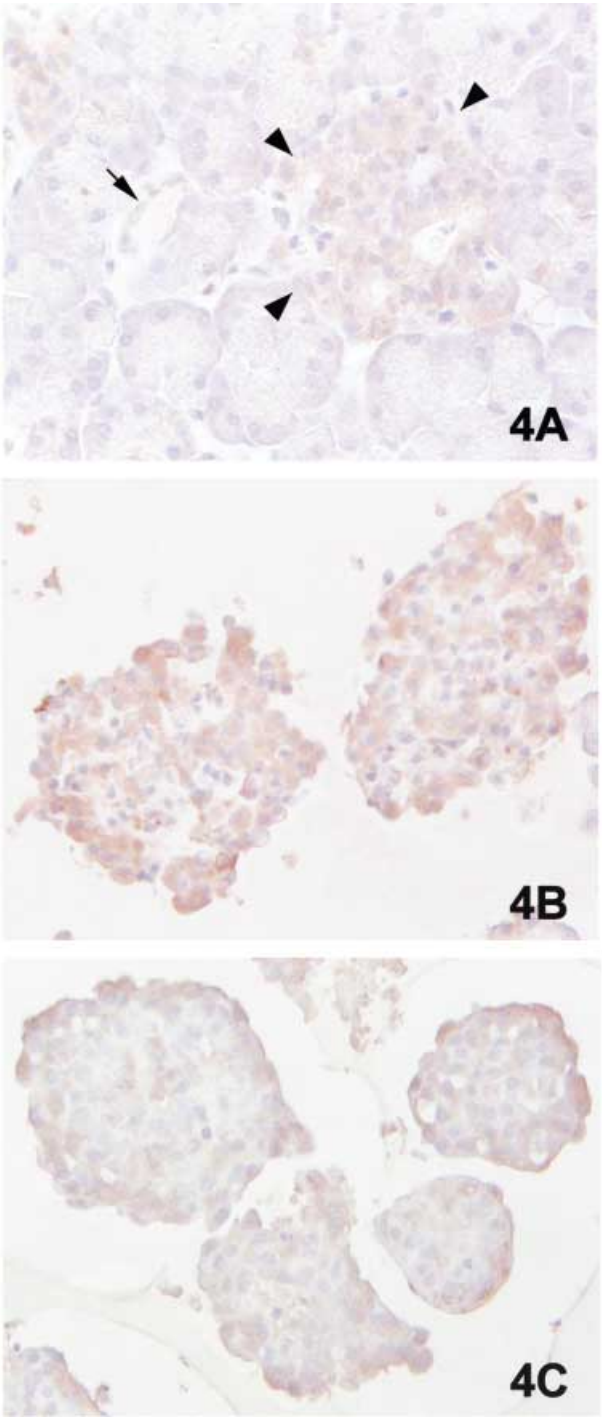


Figure 4 Immunocytochemical staining for integrin α5 in the canine pancreas (A), in isolated islets on day 0 (B), and in islets from the control group on day 4 (C) of culture. The intensity of integrin α5 staining declined following the period of culture. (arrowheads: islet, arrow: duct; × 400).

expression decreased in all experimental groups during the period of culture. Representative changes are shown for integrin α5 in Fig. 4. Morphometric analysis demonstrated that the integrin α5-positive area in control islets decreased 60% by day 6 compared with day 0 (Fig. 3B). In comparison, the relative size of the area of integrin

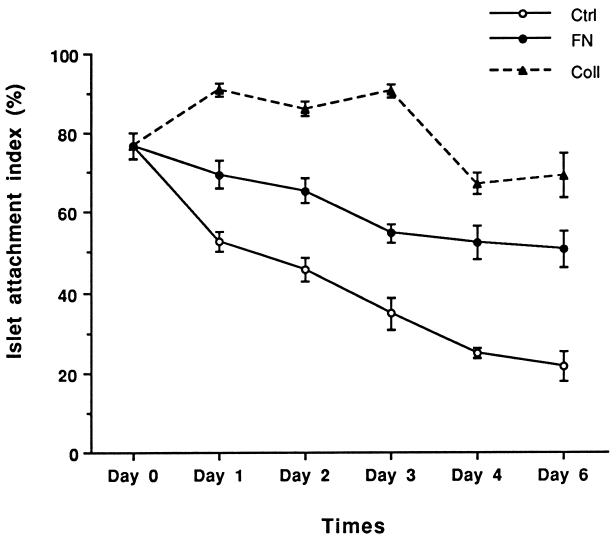


Figure 5 Comparison of canine islet attachment to rat tail collagen in three experimental groups at different time points. Significant differences were observed between controls, the FN and the collagen groups (Coll), at all study points ($P<0{\cdot}05$).

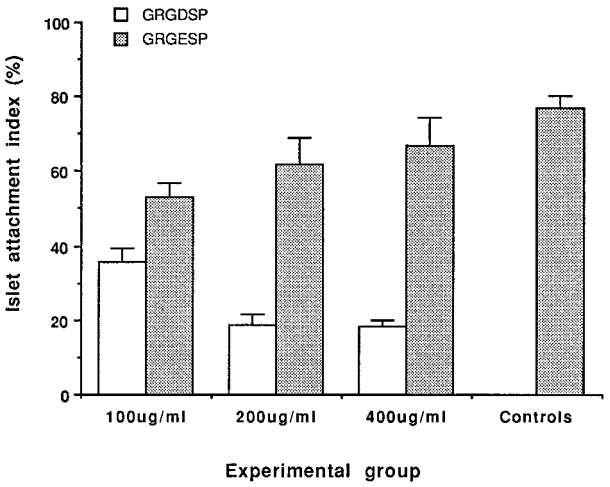


Figure 6 The effect of GRGDSP and GRGESP integrin-binding peptides on adhesion of isolated canine islets to rat tail collagen. Adhesion was partly inhibited by addition of the GRGDSP, but not by GRGESP ($P<0{\cdot}05$).

α5-immunoreactivity in the FN and collagen groups decreased by only 35 and 45% respectively ($P<0{\cdot}01$, Fig. 3B).

As a functional correlate, integrin-mediated adhesion was examined at different time points over the course of the culture period (Fig. 5). Up to 77% of islets adhered to a collagen I matrix immediately after isolation. The attachment index diminished over time, and was significantly lower for control islets (Ctrl) compared with the other two groups, decreasing to 22% at day 6. In comparison, the attachment index for FN- and collagen-cultured islets was 50 and 69% respectively by day 6 ($P<0{\cdot}01$). To eliminate the possibility of nonspecific attachment, the adhesion assay was repeated using a non-coated dish. The data showed less than 5% non-specific attachment in each group. Attachment could be inhibited to a large degree by the addition of the integrin-binding peptide GRGDSP in a concentration dependent manner (Fig. 6). A control peptide (GRGESP) had no influence on islet adhesion.

Insulin content, basal insulin release, and islet response to glucose challenge

Total insulin content of islets in the control group decreased over the course of the culture period (Fig. 7A), dropping more than 65% by day 6. In comparison, FN- and collagen-cultured islets demonstrated an increase in insulin content of 118 and 115% respectively during the initial time period ($P<0{\cdot}05$, Fig. 7A). Thereafter, the insulin content decreased, so that by day 6, it had declined by more than 35 and 80% respectively in comparison to day 0.

Basal 24 h insulin release from islets in the control group decreased during the initial three days of culture by 40% relative to day 0. In comparison, islets cultured with soluble FN, demonstrated a decrease of only 15% (Fig. 7B). Islets maintained on collagen experienced no decrease in basal insulin secretion. Rather a 2·3-fold increase was observed by day 3. When considered together with the dichotomous results of the insulin ICC and ISH for proinsulin (Fig. 2), these findings are most consistent with marked degranulation of beta cells in the collagen group. After day 3, basal insulin release in all three groups declined, but the values for the FN and collagen groups remained higher than the control (Fig. 7B).

To study glucose responsiveness, a glucose challenge study was performed on day 0 and day 2. Islets incubated with 2·2 mM glucose showed a basal insulin release of $25{\cdot}6 \pm 7{\cdot}2$ ng/100 islet/h on day 0. This remained unchanged on day 2. When incubated in the presence of 22 mM glucose plus 50 μM IBMX, islets in the control and collagen groups exhibited a stimulation index of 4 both on day 0 and on day 2 (Fig. 8). In comparison, islets cultured with soluble FN showed a stimulation index of 9 ($P<0{\cdot}002$ vs control and collagen groups, Fig. 8).

Analysis of islet cell death

The apoptotic index for islet cells in each group, as determined by TUNEL staining, is shown in Fig. 9. The index increased in all groups over time. Control islets, however, had an apoptotic index that was significantly higher in comparison to either the FN or collagen groups, reaching a value on day 6 that was 3·6-fold higher than on day 0. In fact, the apoptotic index was significantly greater for control islets at all time points studied ($P<0{\cdot}05$, Fig. 9).

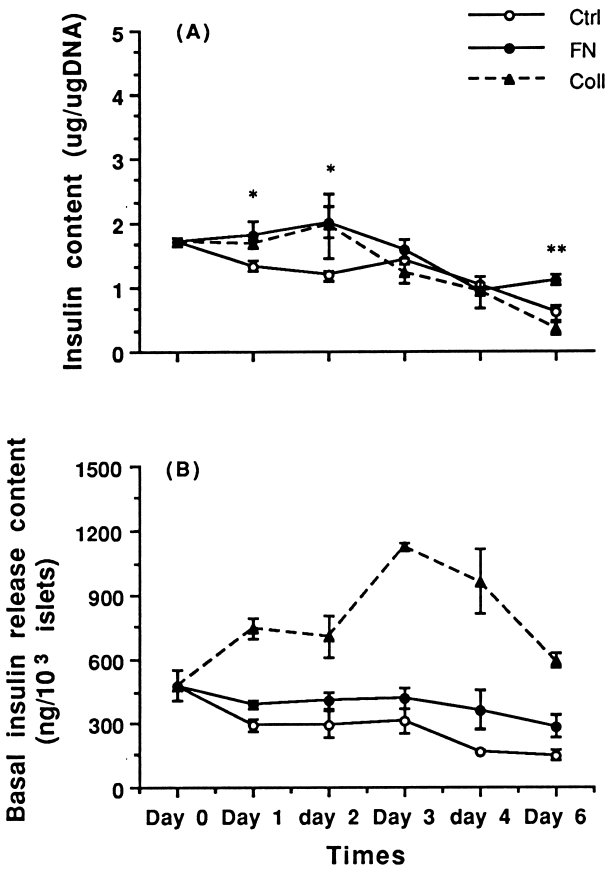


Figure 7 Insulin content (A) and basal 24 h insulin release (B) from cultured canine islets at different time points. Significant differences in the basal insulin release are shown between the collagen (Coll) group and the control and FN groups ($P<0{\cdot}01$). *$P<0{\cdot}05$, **$P<0{\cdot}01$.

Double labeling for TUNEL and insulin or glucagon was used to identify the cell types undergoing apoptosis (Fig. 10). Most of these cells were determined to be beta cells, based on their predominantly central location within the islet and the co-localization of insulin to many of the TUNEL-positive cells. Of course many TUNEL-positive cells no longer demonstrated a cytoplasm and therefore would not be expected to stain for islet cell hormones. However, these observations are in keeping with the results of ISH and ICC, which indicated that the beta-cell area per islet decreased over. In comparison, the glucagon-immunoreactive area per islet remained relatively constant throughout the study period.

Discussion

Islet transplantation is associated with a high rate of early graft failure, manifested by marginal or completely absent C-peptide production and resistance of insulin dependence a problem which remains poorly understood. In this study, we provide for the first time, detailed information on the disruption of the islet–matrix relationship following canine islet isolation, and correlate the findings with both the loss of normal beta-cell function and the onset of islet cell death. We also provide evidence that it may be possible to intervene to prevent or diminish these adverse outcomes by a strategy that seeks to re-establish the islet–matrix relationship.

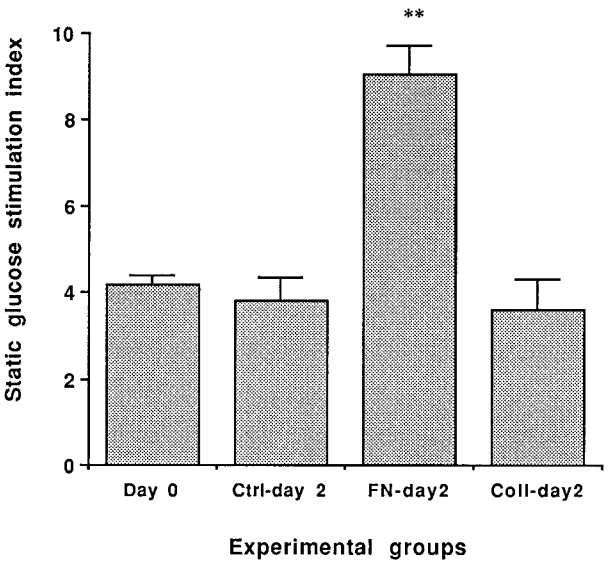


Figure 8 Static glucose stimulation study in isolated canine islets on day 0 and day 2 for the three experimental groups. **$P<0{\cdot}002$.

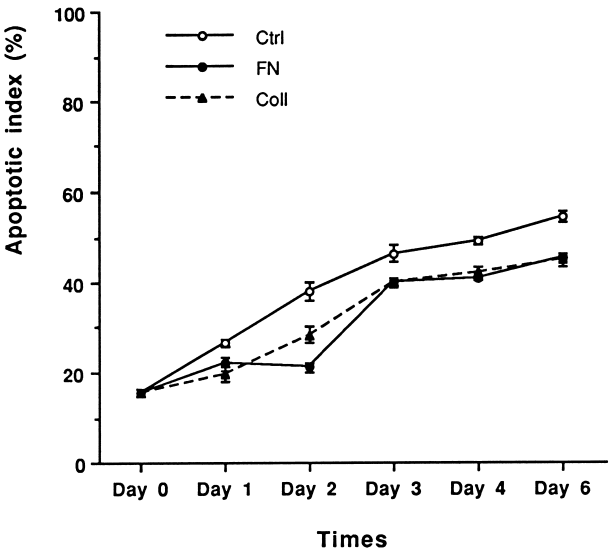


Figure 9 The apoptotic index cells for cultured canine islets at different time points. Significant differences are shown between controls and both the FN and collagen (Coll) groups at all time points ($P<0{\cdot}05$).

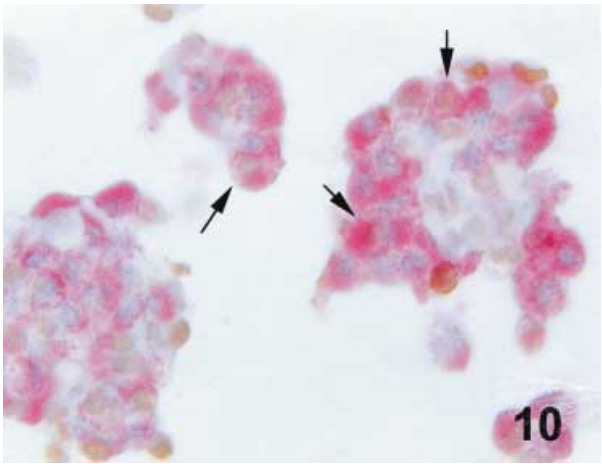


Figure 10 Double staining for TUNEL and insulin of canine islets on day 0. Arrows indicate a beta cell with positive TUNEL staining (brown color). × 640

Enzymatic digestion of the pancreas, the cornerstone of islet isolation, results in the loss of the peri-insular basement membrane (BM). This appeared to be an obligatory loss, since the digestion process is wholly uncontrolled. BM is one of the two forms of ECM, the other being the interstitial matrix (Ingber 1993). ECM components have long been recognized as adhesive and support structures (Schnaper & Kleinman 1993), however ECM also plays a significant role in regulating the behavior of cells. Matrix proteins engender changes in cell shape and movement, bind growth factors, and facilitate cell–cell and cell–matrix interactions.

While a variable loss of the peri-insular BM has already been reported (Gray & Leow 1992, Wang *et al.* 1999), the effect on islet cell survival has not hitherto been considered. For instance, degradation of ECM is known to be an early and critical event in organ regression in several systems (Meredith *et al.* 1993). Moreover, local disruption of ECM results in apoptosis in adjacent cells, whereas survival is promoted by allowing cells to adhere (Frisch & Francis 1994, Chen *et al.* 1997). It is pertinent, therefore, that we observed previously (Paraskevas *et al.* 1997) that islet cells undergo apoptosis following human islet isolation, while others have suggested that this may continue following transplantation as well (Davalli *et al.* 1995, 1996). In this study, apoptosis of beta cells occurred in a like manner following canine islet isolation, and the apoptotic index was greatest for islets not exposed to matrix proteins. This was not an unexpected finding, because islets cultured either on or within solid matrices exhibit improved survival or function (Lucas-Clerc *et al.* 1993, Brendel *et al.* 1994). The protection against apoptosis, afforded by exposure to a matrix protein, however, lasted only 3 days. This indicates that the chosen conditions remain to be further optimized, as additional factors may also be involved. In this regard, the presence of ECM alone may not be sufficient to promote survival, for it is well established that cell survival actually requires integrin-mediated adhesion to matrix proteins (Metrakos *et al.* 1994) for the survival signal to be transduced into the cell.

Integrin expression on canine islet cells has only recently been reported (Wang *et al.* 1999). We observed that canine islet cells express integrins α3, α5, αV. In fact, the distribution of integrins α3 and α5 co-localize most closely with the distribution of insulin immunoreactivity. These particular integrins are known to bind laminin, collagen and FN (Ruoslahti & Reed 1994), and therefore could be implicated in matrix-dependent signal transduction in canine islet cells. The results of the adhesion studies were consistent with a basal level of integrin-mediated adhesion which could be blocked in a dose-dependent fashion by the RGD containing peptide GRGDSP. Although the RGD motif has been associated primarily with FN and laminin, RGD has been reported to have a role in collagen binding as well (Beekman *et al.* 1997, Vitale *et al.* 1997, Grzesik *et al.* 1998). Loss of adhesive function in culture was associated with the loss of integrin expression. The reasons for this remain to be determined, but may relate to changes occurring at the level of the gene in response to alterations in signal transduction (Martins-Green 1997). The observed loss of integrin α5, especially in the control group, may be of considerable importance, because it has been linked to support of cell survival through the upregulation of the expression of bcl-2, an important regulatory protein that inhibits apoptosis (Zhang *et al.* 1995). Finally, the absence of integrin-mediated signals may also contribute to triggering apoptosis through the activation of the stress-activated protein kinase JNK (c-Jun NH_2-terminal kinase), whose downstream effectors are also associated with the induction of apoptosis (Meredith & Schwartz 1997).

ECM also plays an essential role in maintenance of the differentiated state (Lin & Bissell 1993, Ingber 1997). During the period of culture, the relative insulin-immunoreactive area per islet decreased in all groups. This could be explained by: (a) an absolute loss of beta-cell mass, (b) degranulation or failure of hormone storage, (c) decreased insulin synthesis, or (d) a combination of these processes. Comparative analysis of the ICC and ISH findings, together with the data on basal 24 h insulin release and apoptosis suggests the following interpretation. In the control group, the loss of insulin reflected primarily a combination of beta-cell loss secondary to apoptosis and a decrease in insulin synthesis, perhaps at the level of translation. This certainly needs to be further clarified using a more detailed molecular approach. For the collagen group, the loss of insulin appears to result primarily from massive degranulation, coupled perhaps, to a decrease in insulin synthesis. This may reflect a change in phenotypic expression of these cells as they begin to spread out from

the islet and proliferate as a monolayer (Martins-Green 1997). Beta-cell mass and function were the best maintained in the FN group, where in addition to exposure to a matrix protein, islets maintained a spherical three dimensional shape with intact intra-islet cellular relationships. Islets in this group exhibited the smallest discrepancy between immunocytochemical and ISH findings, and the insulin secretory response to a glucose challenge was well preserved. This data clearly highlights, the importance of reconstituting the islet microenvironment following isolation.

In summary, we have characterized the structural, morphologic and functional alterations in the canine islet immediately following isolation and during *in vitro* culture. There were three principal findings: First, the cell–matrix relationship is disrupted by the dissolution of the peri-insular BM and by a loss of integrin expression and function. Secondly, beta-cell apoptosis is induced and appears to be progressive. Thirdly, depending on the culture environment, beta-cell function is variably affected at several levels. This study is unique and contributes new and important information to the field of islet cell biology. If these findings are confirmed, then new strategies that take advantage of the pharmacologic manipulation of the cell–matrix relationship may need to be developed in order to improve the outcome of islet transplantation.

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