Signals for death and differentiation: a two-step mechanism for *in vitro* transformation of adult islets of Langerhans to duct epithelial structures

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Abstract

Phenotypic change of adult pancreatic islets has been implicated in the development of certain pancreatic cancers and in islet transplant failure. The aim of this study was to characterize intracellular events that mediate changes in adult islet phenotype. Using an *in vitro* islet-to-duct transformation model, canine islets were induced to undergo phenotypic transformation to duct-like epithelial structures through a two-stage process. Stage one was characterized by widespread islet cell apoptosis associated with the formation of cavitary spaces within the islets. During this stage, c-Jun N-terminal regulated kinase (JNK) and caspase-3 activities were elevated, while extracellular signal-regulated kinase (ERK) and Akt activities were decreased. The second stage of the process was characterized by an inversion in the balance in activity between these signal transduction pathways and by a concomitant decrease in apoptosis. The transformed islets were no longer immunoreactive for islet cell hormones, but expressed the duct epithelial cell marker CK-AE1/AE3. In contrast to islet cells, these duct epithelial cells were highly proliferative. To clarify the role of the identified changes in signal transduction events, we performed additional studies using pharmacological inhibitors of enzyme activity and demonstrated that inhibition of JNK and caspase-3 activity prevented cystic transformation. Our results indicate that the balance in signaling activity between ERK/Akt and JNK/caspase-3 appears to be an important regulator of islet cell death and differentiation.

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Abbreviations: ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal regulated kinase; PI3K, phosphatidylinositol 3-kinase; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); BrdU, 5-bromo-2' deoxyuridine

Introduction

In the adult pancreas, the endocrine cells that comprise the islets of Langerhans have long been regarded as a stable population of terminally differentiated cells.¹ In comparison, it is well known that other pancreatic cells, the acinar and ductal cells, exhibit plasticity.^{2–4} While recent studies have begun to explore the potential of adult islet plasticity,^{5–7} the cellular and molecular events that regulate this process remain to be elucidated.⁸

An appropriate model for the study of islet plasticity will provide an understanding of mechanisms underlying adult islet cell differentiation. Such a model would be useful for the investigation of a variety of pancreatic pathologies, including many pancreatic cancers, where the cell of origin remains to be identified.⁷ In addition, this model would allow the investigation of changes in islet cell phenotype following islet isolation. These changes in the differentiated state of isolated islets prior to and following transplantation are suggested to lead to the failure of islets to restore glucohomeostasis in a significant population of transplant recipients.^{9–13} This model could, therefore, provide valuable insight into the control of adult islet cell differentiation.

We have previously described an *in vitro* model of the transformation of freshly isolated adult human islets into duct-like epithelial structures.^{5,14} In this model, isolated islets are embedded into a type-1 collagen matrix and supplemented with a defined media. This phenotypic change appears to involve a two-stage process entailing islet cell loss and a change in cell phenotype. Hence, our *in vitro* system offers an excellent opportunity to understand the complex events that are involved in mediating adult islet cell death and differentiation.

In many cell types, phenotypic stability and survival is conferred by trophic factors present in the microenvironment, including extracellular matrix (ECM) proteins such as collagen-I and laminin,^{2,15,16} as well as endogenous growth factors including FGF-2 and TGF- α and - β .^{17,18} The isolation and purification of islets from the adult pancreas leads to the destruction of the ECM and a disruption of potentially important islet–matrix and islet–acinar cellular relations.^{8,17–19} Intracellular signal transduction pathways that mediate the

effects of these trophic factors have been associated with the processes of cell death and differentiation.^{20,21} Of these, among the better characterized signaling pathways are the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK) and c-Jun N-terminal regulated kinase (JNK) as well as the serine/threonine kinase Akt.^{20–26}

Activation of the ERK and PI3-K/Akt signaling pathways have been demonstrated to result in the survival and differentiation of a number of cell types.^{21,23,25,26} Alternatively, activation of JNK and caspase-3 has been associated with the induction of programmed cell death in several cell types.^{22,23,25,27} Moreover, it is the dynamic balance between activities in the ERK/Akt and the JNK/caspase-3 signal transduction pathways that is recognized as a critical determinant of cell differentiation, proliferation and apoptosis.^{23–26,28}

In this study, we demonstrate that islet-to-duct transformation is characterized by a two-stage process involving islet cell apoptosis and a change in the differentiated state of the islet. We report that the relative balance between ERK/Akt activities and those of JNK/caspase-3 appears to define and mediate these two stages in this model of islet cell death and differentiation.

Results

Islet-to-duct-like transformation

Freshly isolated islets of Langerhans (>95% purity) are solid spheroid structures as visualized under the inverted microscope (Figure 1a). By immunohistochemistry (IHC), insulin immunoreactive β cells comprised the majority of the islet tissue (~85%) immediately following islet isolation. Glucagon immunoreactive α cells and somatostatin immunoreactive δ cells surrounded the inner β -cell mass of the islets, together accounting for approximately 15% of the islet tissue (data not shown). From Day 3 onwards, the islets underwent cystic transformation to duct-like epithelial structures (Figures 1c and e). This process was first observed in the mantle region of the islets and progressed until solid islets had been completely replaced by cystic structures.

This change from an endocrine to a duct-like epithelial phenotype was associated with a significant decline in the presence of islet cell hormones, as indicated by IHC. The area of insulin immunoreactive tissue, as determined by computer-aided morphometric analysis, was greatest on Day 0, falling 83% by Day 3 in culture ($P < 0.01$) (Figure 2a). Similar decreases were also observed in glucagon (69% $P < 0.01$) and somatostatin (80% $P < 0.01$) immunoreactive tissue areas (Figures 2b and c). Islet cell hormone immunoreactivities continued to decline to barely detectable levels by Day 12 in culture.

Histologically, the cystic structures were comprised of cuboidal and low-columnar duct-like epithelial cells (Figures 1d and f). In order to better characterize these cells, we analyzed cytokeratin (CK) immunoreactivity as a marker of duct epithelial cell phenotype. CK-AE1/AE3 immunoreactive cells were not identified in islets at Day 0 (Figure 3). With the onset of cystic transformation, the cells that lined the cystic spaces demonstrated CK-AE1/AE3 immunoreactivity. By Day

Figure 1 Representative photomicrographs of islet-to-duct transformation taken after islet isolation (a, b), at Day 3 (c, d) and at Day 6 (e, f) ($\times 100$). Morphological changes are depicted by inverted micrographs (a, c, e) and histological changes are depicted by Hematoxylin & eosin (H&E) staining (b, d, f) ($\times 100$). The formation of cavitory spaces within islet structures is apparent by Day 3 and continues until Day 6 when cystic transformation is complete

6, more than 95% of islets had transformed into cystic structures that were comprised of cells of which over 90% were CK-AE1/AE3 immunoreactive. The apparent loss of cells during the cystic transformation led us to investigate the occurrence of programmed cell death.

Islet to duct-like transformation involves cell death followed by proliferation

Apoptosis was most apparent during the initial 3 days in culture, as demonstrated by a programmed cell death-specific enzyme-linked immunosorbent assay (ELISA) (Figure 4a). In comparison with islets assessed at Day 0, there was a 57% decrease in apoptosis (38 ± 2 nm/ μ gDNA *versus* 16 ± 1) on Day 3 in culture ($P < 0.01$). On Day 6, there was a further 42% decrease in apoptosis (10 ± 1 nm/ μ gDNA) ($P < 0.01$) and on Day 12, this value had decreased another 20% (7.4 ± 1 nm/ μ gDNA) ($P < 0.05$).

To determine which cells undergo apoptosis, colabeling for insulin, glucagon or somatostatin and for TUNEL (terminal

Figure 2 Decline in islet cell hormone immunoreactivity during islet-to-duct transformation, as visualized by immunohistochemistry. The ratio of insulin (a), glucagon (b), and somatostatin (c) immunoreactive area as a percentage of total tissue area was quantified by computer-aided morphometric analysis at different times in culture. Significant differences are shown between all consecutive time points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Representative photomicrographs demonstrating islet cell hormone immunoreactivity at Day 0 and Day 12 are shown adjacent to each graph ($\times 100$)

Figure 3 Increase in immunoreactivity of the duct epithelial cell marker CK-AE1/AE3 during islet-to-duct transformation as visualized by immunohistochemistry. (a) Micrographs representative of CK-AE1/AE3 immunoreactivity after islet isolation (Day 0) and at Day 12 ($\times 100$). (b) The ratio of CK-AE1/AE3 immunoreactive cells to the total numbers of cell present was determined at different times in culture. Significant increases are shown between various consecutive time points (* $P < 0.01$, ** $P < 0.001$)

deoxynucleotidyl transferase-mediated dUTP nick end labeling) was performed (Figure 4b). Using this approach, it appeared that most of the cells undergoing apoptosis were β cells.

Figure 4 (a) Apoptosis of cells during islet-to-duct transformation as quantified by programmed cell death specific ELISA. Significant decreases in cytoplasmic oligonucleosome enrichment are shown between all consecutive time points (* $P < 0.05$, ** $P < 0.01$). (b) Photomicrographs demonstrating beta cell apoptosis during islet-to-duct transformation as visualized by immunohistochemistry for islet cell hormones (red) and TUNEL assay for apoptotic nuclei (brown) ($\times 100$)

Figure 5 Increase in cell survival during islet-to-duct transformation as quantified by MTT assay. Significant differences are shown at consecutive time points (* $P < 0.05$)

In keeping with this initial wave of apoptosis, islet cell survival was found to be 62% higher ($P < 0.05$) on Day 3 of culture, compared to Day 0 (Figure 5), as determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. On Day 6 of culture, there was a further 30% increase in cell survival ($P < 0.05$).

Expansion of the emerging cystic structures was noted from Day 6 onwards. This may have occurred as a result of increased fluid secretion by the cells lining the cysts and/or by cell proliferation. Hence, we assessed proliferation using Bromo-deoxy-Uridine (BrdU) incorporation. At Day 0, the incorporation of BrdU into islet cells was below detectable levels (Figure 6). On Day 3 of culture, concurrent with the onset of cystic transformation, BrdU incorporation was detected in 23% of the nuclei of single-layered cuboidal epithelial cells, and not in any of the remaining islet cells ($P < 0.001$). By Day 6, when >95% of the islets had transformed into duct-like epithelial structures, this value increased by 58% ($P < 0.01$) to 55%. By Day 12, there was a further increase of 37% ($P < 0.05$), as 87% of all cells in culture had incorporated BrdU into their nuclei as the duct-like epithelial structures continued to proliferate and expand rapidly.

Figure 6 Increase in cell proliferation during islet-to-duct transformation, as visualized by BrdU immunohistochemistry. (a) Representative photomicrographs of BrdU immunoreactivity after islet isolation (Day 0) and at Day 12 ($\times 100$). (b) The ratio of BrdU immunoreactive nuclei to the total number of nuclei present was determined at different times in culture. Significant increases were determined between consecutive time points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Signal transduction events during islet-to-duct transformation

In order to better understand the underlying events mediating the phenotypic switch from endocrine to duct-like epithelial cells, we examined signaling events known to be associated with apoptosis (JNK and caspase-3)^{22,27} and survival, proliferation and differentiation (ERK and PI3-K/Akt)^{21,23,25,26}

On Day 0, JNK and caspase-3 activities (activation relative to expression) were highest in contrast with subsequent time points (Figure 7), as determined by Western blot analysis. This was in keeping with the initial high level of apoptosis previously described. JNK and caspase-3 activities subsequently declined by 61 ($P < 0.01$) and 34% ($P < 0.05$) respectively, by Day 3. There was a further 76 ($P < 0.05$) and 64% ($P < 0.01$) decrease, respectively, by Day 6. JNK and caspase-3 activities persisted to Day 12 in culture.

ERK and Akt activities were highest on Day 0 and subsequently decreased by 92 ($P < 0.001$) and 69% ($P < 0.001$), respectively, by Day 3 (Figure 8). Thereafter, ERK and Akt activities increased significantly throughout the remainder of the culture period. Compared to Day 3, ERK and Akt activities increased 405 ($P < 0.01$) and 98% ($P < 0.01$), respectively, by Day 6, with a further increase of 16 ($P < 0.05$) and 12% ($P < 0.01$), respectively, by Day 12.

To determine the contribution of specific signal transduction events to the cystic transformation of islets, pharmacological inhibitors of proapoptotic events (JNK phosphorylation and caspase-3 cleavage) and prosurvival events (ERK and Akt phosphorylation) were examined (Figures 9,10 respectively, compared to the untreated control. Following 6 or 12 days of culture, there were no significant differences in the level of apoptosis with treatment, though apoptosis was already markedly decreased in comparison to Day 0 controls.

Figure 7 (a) Inset: JNK1/2 phosphorylation (activation) and expression as determined by Western blot analysis. Blots are representative of six independent experiments. Graph: Time course of aggregate JNK1/2 activity (activation normalized to expression) during islet-to-duct transformation as determined by computer-aided densitometric analysis. Significant decreases are shown at various consecutive time points (*** $P < 0.001$). (b) Inset: Caspase-3 cleavage (activation) and expression as determined by Western blot analysis. Blots are representative of six independent experiments. Graph: Time course of caspase-3 activity (activation normalized to expression) during islet-to-duct transformation as determined by computer-aided densitometric analysis. Significant decreases are shown at various consecutive time points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Inhibition of both JNK and caspase-3 activities together caused a 96% decrease on Day 3 (control). Following 6 or 12 days of culture, there were no significant differences in the level of apoptosis with treatment, though apoptosis was already markedly decreased in comparison to Day 0 controls. Inhibition of both JNK and caspase-3 activities together caused a 96% decrease on Day 3 ($P < 0.001$), relative to the untreated control. Thus, the level of apoptosis, which appeared to decrease spontaneously between Day 0 and Day 3, was further diminished by the addition of pharmacologic inhibitors of JNK activation and caspase-3 cleavage. This additional reduction in apoptosis, however, did not appear to reflect any additive or synergistic action of these two inhibitors.

Islet-to-cyst transformation in the presence of these inhibitors was severely impaired. We observed that by Day 3, fewer than 20% of the islets treated with SB20358 formed cystic structures, whereas none of the islets treated with z-VAD-FMK formed cysts. Similarly, none of the islets transformed into cystic structures when both inhibitors were administered together.

Figure 8 (a) Inset: ERK1/2 phosphorylation (activation) and expression as determined by Western blot analysis. Blots are representative of six independent experiments. Graph: Time course of aggregate ERK1/2 activity (activation normalized to expression) during islet-to-duct transformation as determined by computer-aided densitometric analysis. Significant decreases are shown at various consecutive time points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (b) Inset: Akt phosphorylation (activation) and expression as determined by Western blot analysis. Blots are representative of six independent experiments. Graph: Time course of Akt activity (activation normalized to expression) during islet-to-duct transformation as determined by computer-aided densitometric analysis. Significant decreases are shown at various consecutive time points (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

When ERK or Akt activity was inhibited by PD98059 or wortmannin, respectively, the level of apoptosis reached at Day 6 increased by 46 ($P < 0.01$) and 40% ($P < 0.01$), respectively (Figure 10b), in comparison to the untreated controls, while at Day 12, apoptosis, relative to the untreated control, was increased by 71 ($P < 0.01$) and 51% ($P < 0.001$), respectively. Inhibition of both ERK and Akt activities together resulted in a 59% increase in apoptosis on Day 6 ($P < 0.01$) and a 79% increase at Day 12 ($P < 0.01$), relative to control. An additive or synergistic effect was not observed.

With respect to cellular proliferation, ERK inhibition was associated with an 80% decrease in BrdU labeling at Day 6 ($P < 0.001$) and an 84% decrease at Day 12 ($P < 0.001$) (Figure 10c), in comparison to the untreated control. Akt inhibition was associated with a decrease in BrdU labeling by 41% at Day 6 ($P < 0.01$) and by 49% at Day 12 ($P < 0.001$), relative to the untreated control. Inhibition of both ERK and Akt activities together caused a 90% decrease in BrdU labeling at

Figure 9 The pharmacologic inhibitors SB20358, z-VAD-FMK, PD98059 and wortmannin decreased JNK, ERK and Akt phosphorylation and caspase-3 cleavage, respectively, to undetectable levels. Blots are representative of four independent experiments

Day 6 ($P < 0.001$) and a 92% decrease at Day 12 ($P < 0.001$), relative to the uninhibited control, which is indicative of an additive effect.

Previously, it had been reported that Akt activity is elevated in human endothelial cells cultured in type-1 collagen.²⁹ Having already demonstrated that type-1 collagen is necessary for islet-to-duct transformation,^{5,14} and since this process appears to be associated with an increase in Akt activity, we sought to examine the independent effect of type-1 rat tail collagen on Akt activation in adult islets immediately after culture. Compared to freshly isolated islets maintained overnight in basal media alone, the activity of Akt in islets cultured overnight in type-1 collagen with basal media was two-fold greater (Figure 11), suggesting that some of the changes in Akt activity during islet-to-duct transformation may be due to the presence of type-1 collagen.

Discussion

Phenotypic changes in adult pancreatic islets have been implicated in the development and progression of several disease states. Pour *et al.*,³⁰ for example, have suggested that islet-to-duct transformation may play a role in the development of pancreatic ductal-type adenocarcinomas.³⁰ However, the specific cell of origin of these tumors remains to be identified.^{7,31} In addition, the loss of endocrine phenotype, function and survival following islet isolation^{6,9,12,13,32,33} and transplantation⁹⁻¹¹ has been implicated in the failure of islet grafts to restore euglycemia in diabetic transplant recipients. In the present study, we extend our previous work on an *in vitro* model of islet-to-duct transformation^{5,8,14} to the char-

Figure 10 The effect of signal transduction event inhibition on apoptotic cell death, as determined by a programmed cell death ELISA. (a) SB20358 and/or z-VAD-FMK treatment are associated with significant decreases in the level of apoptosis at Day 3. (b) Significant increases in the level of apoptosis at Day 6 and Day 12 were observed after PD98059 and/or wortmannin treatment. (c) PD98059 and/or wortmannin treatment was associated with a significant decrease in cell proliferation at Day 6 and Day 12, as visualized by BrdU immunohistochemistry. Significant differences are shown at various time points (* $P < 0.001$, ** $P < 0.01$, *** $P < 0.05$)

Figure 11 Effects of type-1 collagen on Akt activity (activation normalized to expression). Inset: Akt phosphorylation (activation) and expression as determined by Western blot analysis. Blots are representative of four independent experiments. Graph: Akt activity (activation normalized to expression) was two-fold higher in islets cultured overnight in basal media in comparison with islets cultured overnight in basal media alone (* $P < 0.05$, ** $P < 0.001$)

acterization of the opposing signaling events that underlie this phenotypic switch.

Until now, the mechanisms underlying phenotypic changes in adult pancreatic islets have not been well understood. An *in vitro* model of islet cell plasticity would facilitate the elucidation of putative regulatory mechanisms of islet cell differentiation and the subsequent development of strategies to prevent changes in islet cell phenotype. Hence, the purpose of this study was to delineate the morphological changes and associated signaling events that occur during islet-to-duct differentiation. We demonstrate that this transformation entails a two-stage process mediated by a balance in activity between ERK/Akt and JNK/caspase-3 signal transduction pathways.

The first stage of the transformation process is characterized by islet cell loss through apoptosis. This is followed by a second stage in which there is a phenotypic switch from solid islet to cystic duct-like structures lined by columnar and cuboidal epithelium. This process is reminiscent of the cavitation process observed in the early mouse embryo in which cell loss by apoptosis is followed by the transformation of the solid embryonic ectoderm into a cavity surrounded by columnar epithelium.³⁴ Coucouvanis *et al.*³⁴ have suggested that this cavitation process is comprised of two distinct stages, each with seemingly opposing underlying signals and outcomes.

During the first stage of islet phenotypic transformation, widespread cell loss occurs by apoptosis, a process probably initiated by the islet isolation procedure itself,^{8,32,33} during which critical islet-matrix interactions are disrupted.⁸ Meredith *et al.*^{14,15} and Boudreau *et al.*³⁵ have shown that human endothelial cells, and gut and mammary epithelial cells undergo apoptosis when the cell-matrix relation is perturbed. Moreover, given that the ECM functions to instruct cellular phenotype (reviewed in Streuli³⁶), it is quite possible that the islet-to-duct phenotypic switch that we describe may be initiated, at least in part, by the disruption of the islet-ECM relation. Interestingly, Hisaoka *et al.*³⁷ suggest that the ECM protein laminin may provide guidance for terminal cellular differentiation during pancreatic morphogenesis in rats. Similarly, Menko *et al.*³⁸ found that in the absence of the $\alpha_3\beta_1$ integrin receptor for laminin, the activation of the prosurvival kinase ERK was suppressed and salivary epithelial cells exhibited an altered phenotype.

We observed that islet cell apoptosis led to the formation of cavitory spaces within the islets. This morphological change correlated with the observed patterns of signal transduction activation events such that JNK and caspase-3 activities were increased relative to ERK and Akt, both of which decreased and remained low during this first stage of the transformation process. This is keeping with results described by Davis in which the withdrawal of trophic support induces apoptosis through an elevation in JNK and caspase-3 activities.²² Furthermore, a role for JNK and caspase-3 involvement in the induction of apoptosis has previously been demonstrated in neuronal cells²² as well as in islets immediately after isolation.^{27,32,33}

The reciprocal relation between the proapoptotic signal caspase-3 and the prosurvival signal ERK early in the transformation process may be explained, at least in part, by the ability of caspase-3 to cleave and inactivate Raf-1, an

upstream activator of the ERK signal transduction pathway.³⁹ The data support the notion that it is the suppression of the ERK pathway together with the activation of JNK and caspase-3 that is associated with the widespread occurrence of islet cell apoptosis during the first stage of islet-to-duct transformation. These observations lend further support to the notion that the balance in activity between death and differentiation/proliferation signal transduction pathways is a critical determinant of cell fate.

The imbalance in activity between these opposing sets of signal transduction pathways sharply delineates the first stage of the islet-to-duct transformation from the second. During this latter stage, the observed disappearance of islet hormones can be explained by the preceding wave of islet cell apoptosis and a phenotypic switch of the remaining cells to duct-like epithelial cells, which no longer express islet cell hormones.

In the second stage of islet-to-duct transformation, ERK and Akt activities are increased, relative to the first stage. This increase in signaling through prosurvival pathways occurs coincident with a decline in the activity of caspase-3, a biomarker and executioner of apoptosis.⁴⁰ Hence, it is not surprising that during this second stage of the transformation process, there is a significant reduction in apoptosis. This result may be further explained by the ability of active Akt to inhibit apoptosis by phosphorylation and inactivation of the apoptosis-initiating enzyme caspase-9,⁴¹ the proapoptotic Bcl-2 homolog Bad^{42,43} and the Forkhead family transcription factor FKHRL1, which mediates the transcription of proapoptotic gene products.^{44,45} Levresse *et al.*⁴⁶ have also documented Akt-mediated inhibition of the JNK signal transduction pathway, further highlighting Akt's antiapoptotic actions.⁴⁶ Hong *et al.*²⁶ found that increased activation of PI3-K/Akt and ERK, and inhibition of JNK activation, were necessary and sufficient to prevent apoptosis in cardiac myoblasts,²⁶ while Yoon *et al.*²⁵ reached similar conclusions in adipocytes.²⁵ The shift in the relative balance in these signaling activities has also been shown to regulate cell death and differentiation in primitive neuroectodermal tumors.²⁴

Owing to the complexity of signal transduction networks and the crosstalk that is common between different pathways, we have begun to gather information on the role of other signaling events in this model. For example, elevation of intracellular level of cAMP appears to be necessary for islet-to-duct transformation to occur.¹⁴ Additionally, we have determined in preliminary studies that the onset of cystic transformation is also related in a time-dependent fashion to the upregulation of TGF- β -1 protein and receptor during the first 48 h which is rapidly reversed, suggesting the involvement of a TGF- β -1-mediated autocrine loop.⁴⁷ Thus, it appears that the initial stage of islet-to-duct transformation is mediated by a network of signal transduction events that are interrelated by crosstalk between the respective pathways.^{21–28,46,48}

Of the signaling molecules we studied, Akt is the one that appears to play a major role in islet cell survival and differentiation.^{44,45,29} Hence, an understanding of the factors that regulate Akt activity in isolated islets may prove to be of use in controlling phenotypic stability. Recently, constitutively active Akt has been implicated in the induction and regulation of islet cell differentiation and survival in transgenic mice.^{49,50} This finding, coupled with our observations that elevated Akt

activity regulates changes in cell phenotype, survival and proliferation, suggest that type-1 collagen-mediated Akt activity may be a critical mediator of the differentiation/proliferation signal that characterizes the second stage of our *in vitro* islet-to-duct model.

While cellular transformation is known to be important during embryonic development,^{34,51,52} the significance of its occurrence in adult islets, including involvement in the loss of islet phenotype following transplantation or during pancreatic carcinogenesis, remains to be fully elucidated. Our data suggest that at least part of the basis of stability of cell survival and phenotype in adult islets is regulated by the relative balance in activity between the ERK/Akt and JNK/caspase-3 signal transduction pathways.

Materials and Methods

Islet isolation and purification

Pancreata from six mongrel dogs of both sexes (body weight 25–30 kg, age 2–3 years) were resected under general anesthesia in accordance with the Canadian Council for Animal Care guidelines. Prior to organ removal, pancreatic ducts were cannulated to permit intraductal infusion with Liberase CI (1.25 mg/ml) (Boehringer Mannheim, Indianapolis, IN, USA), according to established protocols.^{53,54} Purification was achieved with the use of a continuous gradient of Ficoll-diatrizoic acid (Seromed-Biochrom KG) in an apheresis system (model 2991, COBE Laboratories) (COBE BCT, Denver, CO, USA).⁵⁵ The purity of the final preparations was greater than 95%, as indicated by dithizone staining of tissues with diameters ranging from 50 to 500 μ m. Immunohistochemistry to detect the presence of amylase and cytokeratin was negative, consistent with the absence of ductal and exocrine tissue (data not shown).

Islet cell culture

Freshly isolated islets were embedded in type-1 rat tail collagen gel.⁵ and cultured in Dulbecco's modified Eagle medium (DMEM/F12) (GIBCO, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), epithelial growth factor (EGF) (100 ng/ml) (Sigma, St Louis, MO, USA) and cholera toxin (CT) (100 ng/ml) (Sigma, MO, USA). Approximately 3000 islets equivalents (IEQ) per group per time point were used. Islets were cultured in 95% air, 5% CO₂ at 37°C, and the medium was changed on alternate days. To determine whether the observed changes in intracellular kinase activity were directly implicated in cell death and differentiation in this model, we employed pharmacological inhibitors of JNK (SB20358 at 50 μ M) (Calbiochem-Novabiochem, San Diego, CA, USA), caspase-3 (z-VAD-FMK at 10 μ M) (Promega Corp., Madison, WI, USA), ERK (PD98059 at 50 μ M) (New England Biolabs, Beverly, MA, USA) and Akt (wortmannin at 100 nM) (Calbiochem-Novabiochem, San Diego, CA, USA). Dose-response curves were established for each inhibitor in order to determine optimal concentrations (data not shown). Representative islets from each group were examined after an overnight recovery following isolation (Day 0) and on Days 3, 6 and 12 in culture using the following investigations.

Islet cell lysis and protein content

Islet samples were spun at 900 rpm for 2 min at 4°C. The pellet was washed twice with ice-cold phosphate-buffered solution (PBS) and then dissolved in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.37 mM NaCl, 1% (v/v)

nonidet P-40, 10% (v/v) glycerol, 0.1 mM sodium orthovanadate and complete protease inhibitor cocktail tablet (Boehringer-Mannheim)). Samples were sonicated and the supernatant was kept for Western blot analysis. The protein content was determined using BIO-RAD Protein Assay Dye Reagent (BIO-RAD, Hercules, CA, USA). Lysates were diluted 6:1 with 6 × Laemmli sample buffer (0.375 M Tris-HCl pH 6.8, 12% w/v SDS, 3% w/v glycerol, 0.2% w/v bromophenol blue, 12% β-mercaptoethanol in ddH₂O) and boiled for 3 min.

Western blot analysis

An equal amount of protein (75 μg) was loaded for each sample onto a 12% polyacrylamide gel run at 100 V for 90 min. Transfer onto nitrocellulose was conducted at 250 mA for 90 min. Membranes were blocked with 2% bovine albumin in washing buffer (25 mM Tris, 150 mM NaCl, 0.05% Tween 20 in ddH₂O). Blocked membranes were then probed with primary antibodies. Anti-Akt and anti-active-Akt (Ser-473) (New England Bio-Labs, Beverly, MA, USA) were used at 1:1000 dilutions. Anti-active-ERK (Santa Cruz Biotechnology, CA, USA), anti-ERK (Promega Corp., Madison, WI, USA) and anti-active-JNK (Promega Corp., Madison, WI, USA) were used at 1:5000. Anti-caspase-3 (active and expression) (New England Bio-Labs, Beverly, MA, USA) and anti-JNK (Santa Cruz Biotechnology, CA, USA) were used at 1:1000. Following primary antibody incubation, blots were washed for 1 h in washing buffer, then incubated for 1 h in anti-rabbit horseradish peroxidase-linked secondary antibody (1:2000) (Amersham Life Sciences Inc., Buckinghamshire, UK). Following another 1 h washing, the blots were developed using the enhanced chemiluminescence system (ECL) (Amersham Life Sciences Inc., Buckinghamshire, UK) and Kodak X-OMAT film (Kodak, Rochester, NY, USA). Membranes were stripped by incubating at 65°C for half an hour in stripping buffer (100 mM β-mercaptoethanol, 2% w/v SDS and 62.5 mM Tris-HCl pH 6.7) and reprobed with primary antibody. Developed blots were scanned on a UMAX Astra 2000P scanner-using Presto! PageManager version 4.20.02 (NewSoft, Inc.).

Morphological analysis

Immunohistochemistry

Tissue was fixed in 4% para-formaldehyde (PFA) and embedded in 3% agarose following a standard protocol of dehydration and paraffin embedding.⁸ Serial sections (thickness 4 μm) were cut from each paraffin block. Consecutive sections were processed for routine histology and immunostained for the pancreatic hormones insulin, glucagon and somatostatin (Biogenex, San Ramon, CA, USA) as well as for CK-AE1/AE3 (Dako Diagnostics Canada, Mississauga, ON, Canada) at 1:100 dilution, using AB complex method (streptavidin-biotin horseradish peroxidase; Dako), as described previously.⁵⁵ The sections were incubated overnight at 4°C with the appropriate primary antibodies. Negative controls involved the omission of the primary antibodies.

Cell death and proliferation

Collagen-embedded tissue cultured in DMEM/F12-CT was harvested using collagenase XI (0.25 mg/ml) (Sigma, Montreal, Que., Canada) and processed using a programmed cell death ELISA (Roche Molecular, Montreal, Que., Canada).⁵⁶ Cells were incubated in lysis buffer for 30 min, and the absorbance of the supernatant, which contained oligonucleosomes, was measured at 405 nm. Variations in sample size were

corrected for by measuring total sample DNA content and standardizing between samples.⁸

To evaluate cell proliferation, cells cultured in DMEM/F12-CT were pre-incubated with 10 μM 5-bromo-2' deoxyuridine (BrdU, Sigma, Montreal, Que., Canada) for 3 h at 37°C. Harvested cells were fixed in 4% PFA as described above. Immunostaining for BrdU was performed using the AB complex method. The sections were pretreated with 0.1% trypsin and 3 N HCl to denature the DNA. A monoclonal anti-BrdU antibody was used at 1:500 dilution (Sigma, Montreal, Que., Canada). To calculate a BrdU labeling index, at least 500 cells were counted for each time point. A BrdU labeling index was determined as the percentage of total cells stained positive for BrdU incorporation.

MTT assay

Aliquots containing 500 IEQ were placed in sterile Eppendorf tubes and 50 μl of stock MTT (5 mg/ml) (Sigma, Montreal, Que., Canada) was added to each sample. The samples were incubated at 37°C for 2 h, washed twice with cold PBS and lysed with 200 μl of DMSO (Sigma, Montreal, Que., Canada). Two 100 μl aliquots from each sample were loaded onto a 96-well plate and the absorbance was measured at 595 nm using a Benchmark Microplate Reader (BIO-RAD, Hercules, CA, USA). Data from subsequent time points were expressed as IEQ by calibrating the assay using the absorbance of 500 IEQ immediately following isolation. Each experiment was performed in triplicate.

Statistical analysis

The scanned Western blots were semiquantified using Gel-Pro Analyzer version 3.0 (Media Cybernetics, LP). All experiments were performed a minimum of four times from separate islet isolations. Statistical analysis was performed using the Student's *t*-test with significance defined as *P* < 0.05.

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