Pancreatic islet cell survival following islet isolation: the role of cellular interactions in the pancreas

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Abstract

The purpose of this study was to characterize the trophic effect of pancreatic duct cells on the islets of Langerhans. Ductal epithelium and islets were isolated from hamster pancreata. In addition, duct-conditioned medium (DCM) was prepared from primary duct cultures that had been passaged twice to remove other cellular elements. Three experimental groups were then established: Group 1, 100 islets alone; Group 2, 100 islets+80 duct fragments; and Group 3, 100 islets in 25% DCM. All tissues were embedded in rat tail collagen for up to 12 days and the influence of pancreatic ductal epithelium on islet cell survival was examined. By day 12, 20·6 ± 3·0% (S.E.M.) of the islets cultured alone developed central necrosis, compared with 6·7 ± 2·0% of the islets co-cultured with ducts and 5·6 ± 1·5% of the islets cultured in DCM ($P<0·05$). The presence of apoptotic cell death was determined by a TdT-mediated dUTP-biotin nick end labelling (TUNEL) assay and by a specific cell death ELISA. DNA fragmentation in islets cultured alone was significantly increased compared with islets cultured either in the presence of duct epithelium or in DCM ($P<0·05$). More than 80% of TUNEL-positive cells were situated in the inner 80% of the islet area, suggesting that most were β-cells. DCM was analysed for known growth factors. The presence of a large amount of IGF-II (34 ng/ml) and a much smaller quantity of nerve growth factor (4 ng/ml) was identified. When the apoptosis studies were repeated to compare islets alone, islets+DCM and islets+IGF-II, the cell death ELISA indicated that IGF-II produced the same beneficial result as DCM when compared with islets cultured alone.

We conclude that pancreatic ductal epithelium promotes islet cell survival. This effect appears to be mediated in a paracrine manner by the release of IGF-II from cells in the ductal epithelium.

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Introduction

After more than 20 years of concerted research and over 300 attempts at human islet transplantation, the achievement of insulin independence remains elusive (Socci *et al.* 1991, Warnock *et al.* 1992, Hering *et al.* 1995).

It is perhaps significant that the major emphasis on islet transplantation has been the enhancement of the purity of the islet preparation to promote engraftment and reduce immunogenicity (Scharp & Lacy 1989). The emphasis on purity presupposes that islet survival is independent of other cellular and matrix elements of the pancreas, yet this hypothesis has never been tested. Therefore the loss of trophic support as a cause of islet graft failure, because of the absence of a suitable microenvironment, needs to be explored.

It is known, for example, that a connection between pancreatic duct epithelial cells and islets is established at an early stage of pancreatic morphogenesis (Githens 1993). Endocrine cells develop by a budding process from embryonic duct-like cells and this process leads to the formation of primitive islets in the mesenchyme adjacent to the ducts (Githens 1993). The final stage of phenotypic expression and cellular differentiation may in fact be controlled by the proximity of cells to a local stimulus originating in the pancreas itself (Fell & Grobstein 1968, Crick 1970). In this regard, the role of cell–cell and cell–matrix interactions in cell development and

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differentiation in the pancreas appears to be of particular importance (Pictet *et al.* 1975, Spooner *et al.* 1977, Montesano *et al.* 1983, Dudek *et al.* 1991). Possible mechanisms of action include secretion of an inducing factor, information exchange through cell–cell contact, and production of extracellular matrix that contains a critical trophic factor.

We have demonstrated that the cellular interrelationships which exist in the pancreas during embryological development are subsequently conserved, and that islet neogenesis can be induced in the adult pancreas (Rosenberg *et al.* 1990, 1992). Moreover, we have already reported that duct epithelial cells appear to secrete a soluble factor(s) that exert(s) a proliferative effect on fully mature islet cells (Metrakos *et al.* 1993). In the present study, we extend our original observations to describe the influence of pancreatic duct epithelium on islet cell survival *in vitro*, and characterize for the first time the specific growth factors involved in an islet–duct paracrine interaction.

Materials and Methods

Animals

Experiments were carried out with 8-week-old female Syrian golden hamsters, 85 g body weight (Charles River, St Jean, QC, Canada), and Canadian Council for Animal Care guidelines for the care and use of experimental animals were strictly followed throughout the study.

Duct isolation

The hamster pancreatic duct isolation was performed with collagenase XI solution (1 mg/ml) (Sigma Chemical Co., St Louis, MO, USA) as previously described (Yuan *et al.* 1995). Digestion was carried out in a stationary waterbath at 37 °C for 30 min, followed by a 10 s dispersion by vortexing. After washing, the digest was filtered through a 94 μm steel mesh filter (Bellco Glass, Vineland, NJ, USA). The retained fragments were harvested by rinsing the inverted filter. The tissues were embedded into neutralized rat tail collagen according to Richards *et al.* (1983) and cultured in 2 ml Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Sigma) supplemented with 10% NuSerum (Collaborative Research Inc., Bedford, MA, USA), insulin (1 μg/ml, Eli Lilly, Toronto, ON, Canada), dexamethasone (1 g/l), soybean trypsin inhibitor (0·1 mg/ml, Gibco, Burlington, ON, Canada), cholera toxin (CT) (100 ng/ml, Sigma), epidermal growth factor (EGF) (10 ng/ml, Sigma), penicillin (100 U/ml, Gibco) and streptomycin (100 μg/ml, Gibco). After 10–12 days of culture, the collagen gel was dissolved by 0·25 mg/ml collagenase. Primary duct epithelial cysts were harvested, washed and re-embedded as before. This process was repeated twice to produce a culture of intralobular ductules by the 3rd week. The final tertiary duct culture was completely devoid of islets, acini and fibroblasts.

Duct-conditioned medium (DCM)

DCM was prepared from purified tertiary ducts incubated at 37 °C for 3 days in DMEM/F12 without NuSerum, CT, EGF or insulin. The conditioned medium was collected and stored at − 80 °C.

Islet isolation

Islets were isolated from hamster pancreata according to a method previously established in our laboratory (Gray & Morris 1987, Metrakos *et al.* 1992, 1993). In brief, 4 ml collagenase P (Boehringer Mannheim, Laval, QC, Canada) solution at 0·7 mg/ml were slowly introduced into the common bile duct after occlusion of the distal end just proximal to the duodenum. The distended pancreas was excised and the digestion was performed in a waterbath at 37 °C for 30 min. Islet purification was achieved using a two-step, discontinuous density gradient of BSA (Sigma). Islets were collected from the interface between the 1·000 and 1·081 g/ml layer. To ensure 100% purity of the preparation, islets were hand-picked and counted under an inverted microscope (Nikon TMS, Japan). The islets were recovered overnight in RPMI 1640 (Gibco) supplemented with 4% NuSerum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Analysis of islet–duct interaction

Ducts and islets were harvested and embedded in rat tail collagen according to the following experimental design: Group 1 (islets alone – control), 100 islets per culture flask; Group 2 (islets plus ducts), 100 islets plus 80 ducts per flask; and Group 3 (islets plus 25% DCM), 100 islets per flask. Eight flasks were established per group in order to ensure sufficient tissue for all proposed studies.

Each group was initially incubated for 3 days in a baseline medium of DMEM/F12 supplemented with 10% NuSerum. After day 3, the medium was changed to serum-free DMEM/F12 (Groups 1 and 2) or to DMEM/F12 plus 25% DCM (Group 3). The medium was changed every other day, and islets were retrieved from the collagen for study on days 1, 3, 6 and 12. A 12 day period of culture was chosen because pilot studies had indicated that there were no new or additional changes in morphology after this time.

Assessment of islet morphology

***In vivo* microscopy** To calculate the number of islets with central necrosis in each experimental group, inverted

light microscopy was used to monitor morphological changes in the islets throughout the incubation period. The same tissue fields were photographed and counted at 1, 3, 6, 8 and 12 days. Necrosis at the centre of an islet was characterized by a zone of very dark cells that was sharply demarcated from the surrounding viable islet tissue. The number of islets with central necrosis was counted and expressed as a percentage of the total. Islet size (μm) and the size of the area of necrosis were determined using a calibrated graticule mounted in the eyepiece of the microscope.

Histology Culture dishes were sampled on days 1, 6 and 12. The tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections (5 μm thick and 50 μm apart) were cut from each block, stained with haematoxylin and eosin (H&E) or toluidine blue and then processed further for routine light microscopy.

Immunocytochemistry Consecutive 5 μm sections cut from the tissue block were immunostained for islet cell hormones (anti-human insulin antibody or rabbit polyclonal anti-glucagon or anti-somatostatin antibodies at a dilution of 1:100, Dako Corp., CA, USA), using the streptavidin–biotin complex method as described previously (Wang *et al.* 1997). The islet hormones were detected using naphtol-AS-MX-phosphate/fast red TR salt as chromagen to obtain a red reaction product. Negative controls were tissues processed with non-immune serum substituting for the primary antibodies. To identify the presence of apoptotic cells, the tissues were co-processed for the TdT-mediated dUTP-biotin nick end labelling (TUNEL) reaction (see below).

Detection of apoptotic cell death

After a 12 day incubation period, the media were removed and 3 ml collagenase XI (0·25 mg/ml) were added to each flask for 40 min. The islets were hand-picked and washed three times in Hank's buffered salt solution (Gibco). The presence of DNA fragmentation, a characteristic feature of apoptosis, was determined using a cell death detection ELISA (Boehringer Mannheim). The results were expressed as absorbance at 405 nm and corrected for total DNA content. DNA was measured by fluorometry using disbenzimidazole (Hoechst 33258, Sigma) as described (Labarca & Pagen 1987).

To identify which cells in the islet were undergoing apoptosis, a TUNEL assay was employed. Islets were fixed in 4% paraformaldehyde, embedded in paraffin and 5 μm sections cut and pretreated with 0·1% trypsin buffer at 37 ° for 5 min. The *In Situ* Cell Death Detection Kit (Boehringer Mannheim) was used for the labelling of apoptotic cells and the sections were developed with diaminobenzidine (Sigma). To reduce non-specific labelling the anti-fluorescent antibody conjugated with horse-radish peroxidase was diluted 1:5 in TBS buffer. Approximately 800 cells from ten islets were counted in each group and an apoptotic index (per cent of labelled cells) was calculated.

Analysis of culture medium for growth factors

Samples of DCM were subjected to acid gel chromatography on Sephadex G50 to separate insulin-like growth factor (IGF)-binding proteins prior to performing specific RIAs for IGF-I and IGF-II. These assays were carried out as described by Hill (1990).

Assay for nerve growth factor (NGF) was performed using an ELISA kit from Boehringer Mannheim. Mouse NGF was used as standard. The antibody specifically reacts with the β-subunit of NGF, and cross reacts with hamster tissues.

Analysis of the effect of IGF-II

The islets were embedded in rat tail collagen and cultured in DMEM/F12 supplemented with 10% NuSerum. After 3 days in culture, three experimental groups were designed for this study: Group 1 (control), islets cultured in DMEM/F12; Group 2, islets cultured in DMEM/F12+ 25% DCM; and Group 3, islets cultured in DMEM/F12+ 34 ng/ml IGF-II. The islets were retrieved from the collagen following 9 days in culture with these media.

Statistical analysis

The results obtained for all culture flasks within a group were pooled and expressed as mean ± S.E.M. The differences between the three experimental groups with respect to the presence of central necrosis, cytoplasmic DNA fragmentation and apoptotic index were analysed by unpaired Student's *t*-test. The relationship between islet size and the presence or absence of central necrosis, and the relationship between islet size and the size of the area of necrosis, were determined by Chi-square analysis. Except for the IGF-II apoptosis study, all experiments were performed twice and the results were averaged, with P values less than 0·05 considered significant.

Results

Characteristics of islets and ducts following isolation

The average number of islets isolated was 475 ± 108 per pancreas. Immediately after isolation, islet purity, as determined by dithazone staining, ranged from 80 to 95%, but this reached nearly 100% on the second day following the final transfer of islets onto the culture dishes. Islet diameter ranged from 50 to 300 μm, with the majority measuring 100–200 μm. Under the inverted microscope, freshly isolated islets had a smooth appearance with slightly

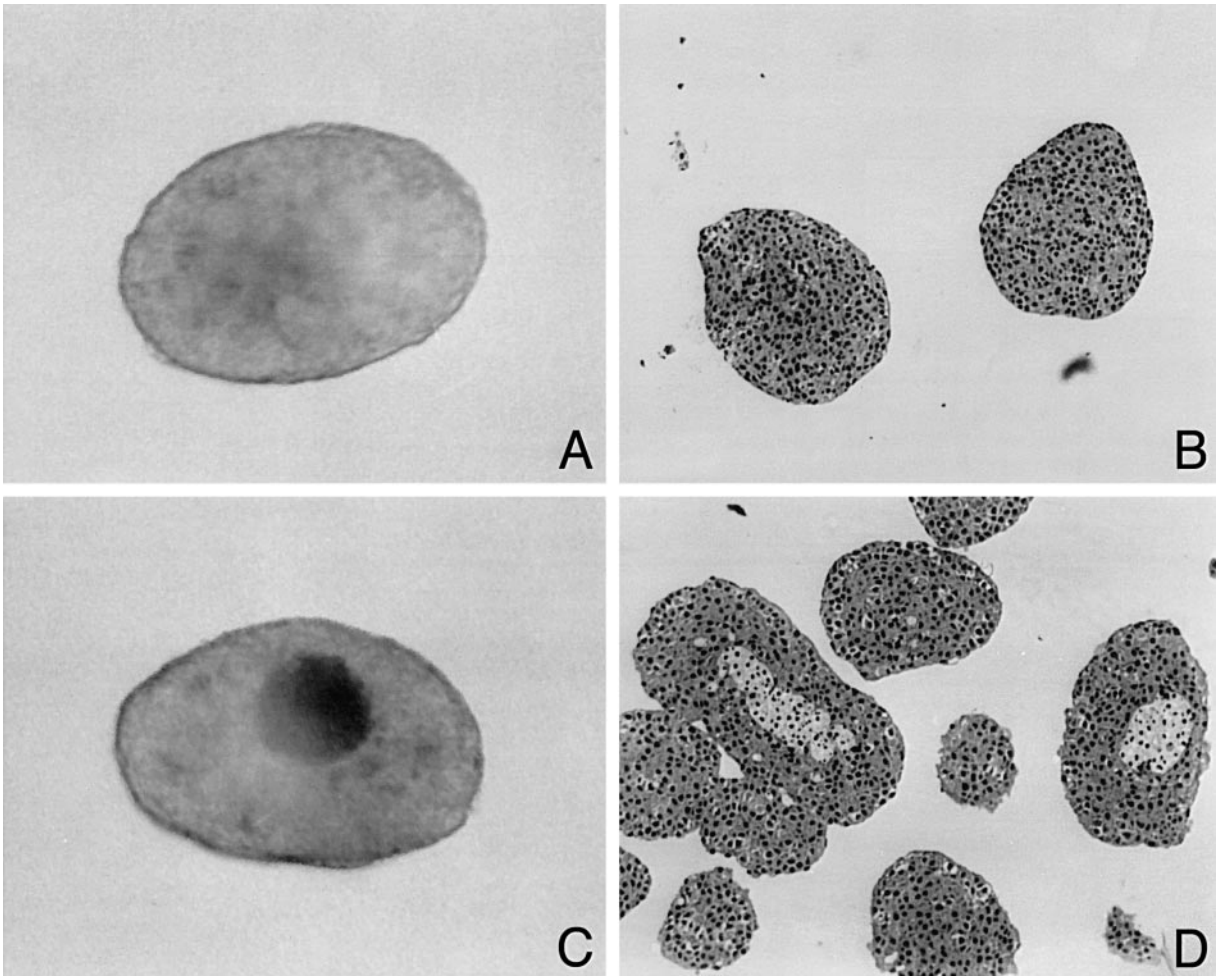

Figure 1 (A) Time-lapsed photograph (× 100) of an islet of Langerhans, embedded in collagen, on day 1. Note the absence of central necrosis. (B) Light micrograph of islets (H&E; × 156) cultured for 1 day in the absence of pancreatic ductal epithelium showing normal islet morphology. (C) Time-lapsed photograph (× 100) of the same islet shown in (A), day 6. Note the presence of a dark central area of cell necrosis. (D) Light micrograph (H&E; × 156) of islets cultured for 6 days in the absence of pancreatic ductal epithelium. Some islets show development of central necrosis, while others appear normal.

irregular borders. After overnight incubation, the islets recovered a more regular spherical shape with well-defined smooth borders.

At the start of the study, the mean islet diameter was similar in each of the three treatment groups – islets alone (161 ± 34 μm) vs islets+ducts (164 ± 61 μm) vs islets+DCM (155 ± 51 μm).

More than 2000 duct epithelial cysts were isolated per gram of pancreatic tissue. Following two passages, cultures of pure duct epithelial cysts were obtained, with the lining wall composed of a single layer of cubical or flattened epithelium.

Analysis of morphological changes

After 1 day, the islets in each group had a normal morphological appearance. The islet cells were compact, regularly shaped, with well-defined nuclei and a grainy appearance of the cytoplasm, and there was no evidence of central necrosis (Fig. 1A and B).

After day 3, a central area of very dark tissue with borders sharply demarcating the surrounding islet cells became visible. By routine histology, this central area was shown to be composed of necrotic cells in the islet core. By day 6, extensive areas of necrotic cells in the central islet region were apparent (Fig. 1C and D).

Islets cultured alone had a significant increase in central necrosis ($P<0{\cdot}001$) at all time points when compared with islets in the other two groups (Fig. 2). By day 12, $20{\cdot}6 \pm 3\%$ of the islets cultured alone were necrotic, compared with $6{\cdot}7 \pm 2\%$ of the islets co-cultured with ducts and $5{\cdot}6 \pm 1{\cdot}5\%$ of the islets cultured in DCM. There was no difference between islets in the duct co-culture and DCM groups.

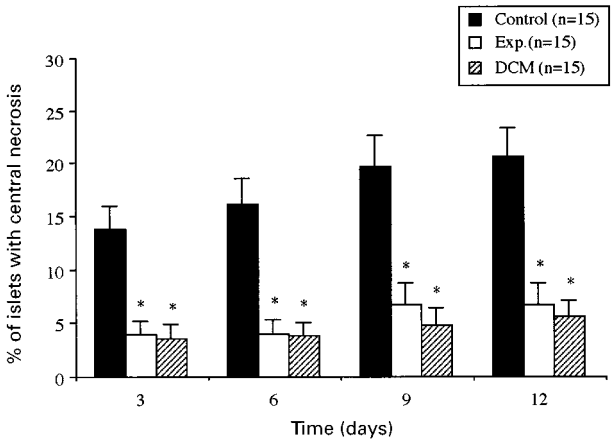

Figure 2 Effect of ductal epithelium and DCM on the development of islet necrosis. Data are means ± S.E.M. *P<0·001.

The presence or absence of central necrosis was associated with islet size (P<0·01), with the area of necrosis being greatest in islets larger than 150 μm. Halfway through the study period, there was no significant difference in the mean diameter of islets with central necrosis whether they were cultured alone (192 ± 49 μm), co-cultured with ducts (209 ± 46 μm) or cultured with DCM (187 ± 42 μm).

Islets in all groups were normally granulated with insulin at the start of the study. Over the course of the study period, islets in Group 1 slowly lost their insulin immunoreactivity compared with islets in Groups 2 and 3. This was most marked at the end of the study on day 12 (Fig. 3A and B).

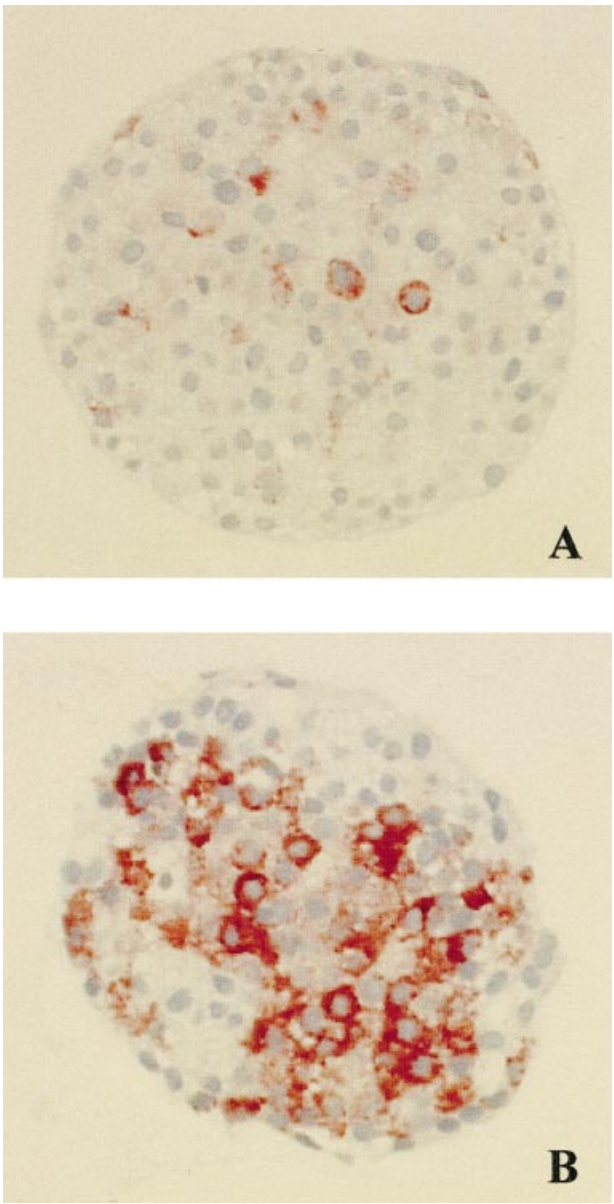

Figure 3 (A) Islet from Group 1 (islets alone) stained for insulin immunoreactivity on day 12. Note the almost complete loss of insulin. (B) Islet from Group 3 (islets in DCM) stained for insulin immunoreactivity on day 12. Note the normal pattern of distribution of insulin in the islet. The same finding was observed for islets cultured with ductal epithelium. (Both × 400.)

Analysis of cell survival

After 12 days of culture, islets in the duct co-culture and DCM groups showed less evidence of apoptosis compared with islets cultured alone. Using the cell death ELISA, the cytoplasmic nucleosome content of islets cultured alone was increased significantly when compared with islets co-cultured with ducts or islets cultured in medium supplemented with DCM (2·3 ± 0·2 versus 0·9 ± 0·5 and 1·1 ± 0·8; P<0·05). The apoptotic index (per cent TUNEL-positive cells) demonstrated a significant difference (P<0·001) between islets cultured alone (60·8 ± 3·9%) compared with those co-cultured with ducts (29·8 ± 8·3%) or in DCM-supplemented medium (38·3 ± 1·5%). Over 80% of TUNEL-positive cells were in the inner 80% of the islet, suggesting that these were primarily β-cells. Double labelling of cells for islet cell hormones confirmed this impression, although as anticipated many TUNEL-positive cells in fact did not show immunoreactivity for any islet cell hormone. By routine histology, the central areas of some islets appeared to also exhibit characteristic features of necrosis, therefore both processes – apoptosis and cell necrosis – were probably responsible for cell loss in the islet core.

Analysis of DCM and the effect of IGF-II

After completion of this initial series of studies, DCM was analysed for growth factors that might be expected to be

secretory products of pancreatic ductal epithelium, based on previous literature reports. Our analysis demonstrated the presence of a small amount of NGF (4 ng/ml) and a much larger amount of IGF-II (34 ng/ml). IGF-I was not identified.

The culture studies were then repeated to compare the outcome by cell death ELISA, for islets cultured alone, to islets+DCM and islets+IGF-II. The results demonstrated that IGF-II could account for the beneficial effect of DCM ($P<0{\cdot}004$) with respect to limiting apoptosis in cultured islets (islets alone, $4{\cdot}9 \pm 0{\cdot}7$; islets+DCM, $2{\cdot}7 \pm 0{\cdot}4$; islets+IGF, $2{\cdot}5 \pm 1{\cdot}0$).

Discussion

We have reported previously that islet–duct co-culture enhances islet cell proliferation *in vitro* (Metrakos *et al.* 1993). The present study was designed to further characterize the trophic interrelationships between pancreatic ducts and islets. The hypothesis tested was that islet isolation and purification procedures disrupt cell–cell interrelationships and that this may lead to a loss of trophic support, and hence to a failure of islet cell survival. A duct–islet interaction was chosen because of the embryological origin of islets from ductal epithelium (Rutter 1980) and because this linkage, established early in pancreatic morphogenesis, appears to be subsequently preserved in the post-natal period (Githens 1993).

When maintained under *in vitro* conditions, islet cells, predominantly in the islet core, were observed to undergo necrotic cell death. This finding is in keeping with a number of previous studies that have documented the occurrence of central necrosis in islets maintained *in vitro* (Ono *et al.* 1979, de Graaff *et al.* 1994, Metrakos *et al.* 1994). In the present study, however, when islets were co-localized with duct epithelium or with the secretory products of duct cells, the occurrence of central islet necrosis was diminished significantly.

This observation may be explained by two possible mechanisms. First, the presence of central necrosis is generally believed to reflect a diffusion-related phenomenon. This is borne out in part by the demonstration that it is generally the larger islets that are more susceptible to this form of cell death. The adverse effect of islet size would certainly be compounded by the removal of a source of trophic support. Re-establishment of such support, however, could be expected to prevent much of the observed necrosis. This was indeed the case when islets were either co-cultured with ducts or supplemented with DCM. The second is the enhanced β-cell proliferation that has been reported in the presence of pancreatic duct epithelium (Metrakos *et al.* 1993). Since the islet core is composed predominantly of β-cells, the ability of these cells to proliferate when co-cultured with ducts, albeit at a low level, could serve to mitigate, at least in part, the tendency for cell loss due to necrosis.

In contradistinction to cell necrosis, apoptosis represents a form of programmed cell death based on a defined molecular mechanism (Oppenheim 1991, Coles *et al.* 1993, Finegood *et al.* 1995), but a variety of environmental factors (Yamada & Ohyama 1988, Golstein *et al.* 1991, Itoh *et al.* 1991, Raff 1992, Levine *et al.* 1993) have also been shown to induce apoptosis. In addition, we were the first to report the occurrence of islet cell apoptosis following routine islet isolation for human islet transplantation (Paraskevas *et al.* 1997*a*).

In the present study, after 12 days of incubation, purified islets sustained a significant increase in apoptotic cell death compared with islets co-cultured with duct cells or those maintained in medium supplemented with DCM. This finding is consistent with the suggestion that islet isolation and purification may trigger apoptosis by removing important trophic factors responsible for the maintenance of islet cell viability (Paraskevas *et al.* 1997*b*,*c*). It remains to be established, however, whether both types of cell death, necrosis and apoptosis, are induced coincidentally by the same set of circumstances, i.e. the withdrawal of trophic factors and the environmental conditions to which the islets are exposed.

These findings suggest that islet isolation and purification, as currently practised, could contribute to an increased rate of islet cell death, and by corollary, to a reduced β-cell mass for transplantation. A number of clinical and experimental studies lend support to our observations. In patients who underwent total pancreatectomy for the relief of pain due to chronic pancreatitis, followed by an intraportal infusion of a relatively small number of unpurified autologous islets, insulin independence was achieved for up to 6 years (Farney *et al.* 1991). On the other hand, following autotransplantation of purified islets in a canine model, most grafts were reported to lose function after only 1 year (Kneteman & Warnock 1990). A similar outcome was obtained even when islets were immunoisolated (Lanza *et al.* 1992, Soon-Shiong *et al.* 1992).

The beneficial effect of DCM in reducing both necrotic and apoptotic cell death is interesting in view of the fact that a number of growth factors have been identified in the pancreas (Pictet & Rutter 1977, Rabinovitch *et al.* 1982, Romanus *et al.* 1985, Hill *et al.* 1987, Polak *et al.* 1993, Scharfmann *et al.* 1993, Wang *et al.* 1993). Growth factors act in a paracrine manner (Goustin *et al.* 1986, Underwood *et al.* 1986) to mediate a broad range of cellular responses (Sporn & Roberts 1987), including extracellular matrix formation, cell proliferation and differentiation. Growth factors may also be inhibitory in almost all situations in which apoptosis is positively stimulated. This suggests that regulation of growth factor levels is not only important in the control of cell proliferation, but also in maintaining viability of cells susceptible to apoptosis (Armato *et al.* 1986, Raff 1992, Collins *et al.* 1994).

The role of these factors in the maintenance of islet cell survival in the pancreas is relatively unexplored, but a role for the IGFs as survival factors has been proposed (Barres *et al.* 1992). IGFs inhibit apoptosis in mammary carcinoma cells, cerebellar granule neurons, ovarian preovulatory follicles, human erythroid colony-forming cells and haematopoietic cells (Geier *et al.* 1992, Muta & Krantz 1993, Chun *et al.* 1994, Galli *et al.* 1995). We recently showed that a developmental apoptosis which occurs in the neonatal rat β-cell population is associated with a loss of local IGF-II expression within the islets (Petrik *et al.* 1998). A functional link existed since the resistance of isolated islets from newborn rats to cytokine-induced apoptosis was reduced when endogenous IGF-II was immunoneutralized, while older islets, which no longer express IGF-II and are killed by cytokines, could be rescued with exogenous IGF-II. It is, therefore, of considerable importance that we have identified IGF-II as a key trophic element in this *in vivo* model of islet cell survival. These data suggest that for success in islet transplantation the activity of survival factors may be fundamental to the long-term maintenance of graft function. As such, it is important that in this study we have identified at least one of the necessary factors and its cell of origin.

In summary, we have demonstrated three important outcomes. First, islet cell death from both necrosis and apoptosis occurred during a brief period of culture following islet isolation and purification. Secondly, there was a trophic interaction between pancreatic duct epithelium and islet cells that produced a significant reduction in islet cell death. Thirdly, this interaction was mediated by duct cell secretion of IGF-II into the culture medium. Further studies will be necessary to clarify the precise nature of the interaction and to identify and purify other possible mediators.

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