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Upregulation of the expression of tight and adherens junction-associated proteins during maturation of neonatal pancreatic islets *in vitro*

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Summary

Cell–cell contacts mediated by intercellular junctions are crucial for proper insulin secretion in the endocrine pancreas. The biochemical composition of the intercellular junctions in this organ and the role of junctional proteins in endocrine pancreatic dysfunctions are still unclear. In this study, we investigated the expression and cellular location of junctional and cytoskeletal proteins in cultured neonatal rat pancreatic islets. Neonatal B-cells had an impaired insulin secretion compared to adult cells. Cultured neonatal islets showed a time-dependent increase in the glucose-induced secretory response. The maturation of B-cells *in vitro* was accompanied by upregulation of the expression of some junctional proteins in islet cells. Neonatal islets cultured for only 24 h showed a low expression and a diffuse cytoplasmic location of the tight junctional proteins occludin and ZO-1 and of the adherens junctional proteins α- and β-catenins, as demonstrated by immunoblotting and immunocytochemistry. Culturing islets for up to 8 days significantly increased the cell expression of these junctional proteins but not of the cytoskeletal proteins vinculin and α-actinin. A translocation of ZO-1 and catenins to the cell–cell contact region, as well as a higher association of F-actin with the intercellular junction, were also observed in neonatal islets following prolonged culturing. ZO-1 and β-catenin were immunolocated in the endocrine pancreas of adult rats indicating that these junctional proteins are also expressed in this organ *in situ*. In conclusion, endocrine pancreatic cells express several junctional proteins that are upregulated following differentiation of the endocrine pancreas *in vitro*.

Introduction

The cells of tissues and organs are connected to each other by specialized, membrane-associated structures known as intercellular junctions (IJs). There are four types of junctions that are structurally and functionally distinct, namely tight junction (TJ), adherens junction, desmosome and gap junction (GJ) (Faquhar & Palade 1963, Gilula 1978). The first three of these junctions are essentially intercellular adhesion devices, whereas GJs consist of connexin-containing channels involved in intercellular communication. Extensive investigation of the molecular biology of IJs has led to the identification of several protein components and to an understanding of the mechanisms regulating their organization and function. Occludin and claudins are specific proteins present in the tight junctional strands that associate with a cytoplasmic protein complex formed by ZO-1, ZO-2, ZO-3, cingulin and other proteins (Zahraoui *et al.* 2000). Adhesion molecules belonging to the cadherin superfamily are the main components of adherens junctions and desmosomes (Geiger & Ayalon 1992). These adhesion glycoproteins are linked to the cytoskeleton through a distinct group of proteins that includes catenins, in the case of the adherens junction, and plakoglobin (also known as γ-catenin) and desmoplakin, in the case of desmosomes (Geiger *et al.* 1995, Burdett 1998, Harington & Syrigos 2000). All IJs and their proteins are dynamic, regulated membrane structures that play an important role in various cellular events, such as migration, differentiation, intercellular communication, cell–cell recognition, cell growth and death (Collares-Buzato *et al.* 1994a, b, 1998a, b, 2001, Allen *et al.* 1996, Gottardi *et al.* 2001, Li *et al.* 2001, Ling *et al.* 2001).

Cell–cell contacts are crucial for a proper secretory response by the endocrine pancreas. The synthesis and release of insulin are markedly altered after dispersion of B-cells *in vitro*, but rapidly recover after cell reaggregation, indicating that the secretory mechanism depends on intercellular interactions within the pancreatic islet (Halban *et al.* 1982, Bosco *et al.* 1989, Pipelleers *et al.* 1994). Ultrastructural analysis has shown that endocrine pancreatic cells interact within the islet through IJs such as TJs, desmo-

somes, adherens-type junctions and GJs (Faquhar & Palade 1963). Among the IJs, GJs have been the most studied in the pancreas. The importance of intercellular contact for normal pancreas functioning has been attributed mainly to the GJs interconnecting the islet cells. Several studies *in vivo* and *in vitro* have demonstrated that insulin secretion stimulated by glucose and other secretagogues is associated with increased GJ-mediated coupling between B-cells, whereas the pharmacological blockage of GJ channels impairs the secretory function of these islet cells (Meda & Orci 1979, Meda *et al.* 1983, 1990, 1991, Collares-Buzato *et al.* 2001). The biochemical composition of the other IJ components in this organ and the role of junctional proteins in endocrine pancreatic dysfunctions are still unclear.

In this work, we investigated the expression and cellular location of junctional and cytoskeletal proteins in cultured pancreatic islets from neonatal rats. Neonatal B-cells have an impaired insulin secretion compared to that of islets from adult rats (Boschero *et al.* 1988, 1993). The maintenance of neonatal islets in culture for prolonged periods results in a time-dependent improvement in the glucose-induced secretory response through a mechanism that is not completely understood (Boschero *et al.* 1993, Crepaldi-Alves *et al.* 1997, Collares-Buzato *et al.* 2001). Thus, cultured neonatal islets provide a useful model for studying the cellular processes underlying the maturation of the pancreatic secretory machine. In addition, such studies may yield results that could form the basis for optimizing the use of cultured neonatal islets or precursor cells for transplantation in the treatment of diabetes. As shown here, endocrine pancreatic cells express several junctional proteins that are upregulated following differentiation of the endocrine pancreas *in vitro*. These findings suggest a possible role for IJs in determining an adequate endocrine function of the pancreas.

Materials and methods

Materials

Cell culture media were supplied by Sigma (St. Louis, MO, USA) and culture medium supplements were purchased from Cultilab or Nutricell (Campinas, SP, Brazil). Sterile plastic materials were purchased from Corning (Corning, NY, USA). Radiolabeled insulin and protein A were obtained from Amersham Biosciences (Cleveland, OH). Primary antibodies, FITC- or TRITC-conjugated secondary antibodies and TRITC-phalloidin were purchased from Sigma, Zymed (San Francisco, CA), Dako (Carpinteria, CA, USA) and Upstate Biotechnology Inc. (New York, NY, USA). All other chemicals and reagents were supplied by Sigma and Merck (Darmstadt, Germany).

Animals

Neonatal male and female Wistar rats (2–48 h old) and adult male Wistar rats (3–4 months old) were obtained from the breeding colony at the State University of Campinas (UNICAMP, Brazil). The animals were housed and subsequently killed to obtain the pancreas in accordance with the guidelines of the institutional Committee for Ethics in Animal Experimentation (UNICAMP).

Islet culture and isolation

Islets from neonatal Wistar rats were cultured as previously described (Collares-Buzato *et al.* 2001). Each neonatal rat pancreas yielded 100–200 islets. Briefly, the islets were cultured for varying periods of time (maximum of 8 days) in sterile Petri dishes at 37 °C in a humidified 5% CO_2/air atmosphere. The culture medium consisted of RPMI-1640 containing 10 mM glucose and supplemented with 5% fetal calf serum (heat inactivated) and 100 IU of penicillin/ml and 100 μg of streptomycin/ml. The culture medium was changed every second day. At the end of the culture period, the islets were collected individually under a dissecting microscope using a micropipette. Some of these islets were used to determine the levels of insulin secretion and the remaining ones were processed for immunocytochemistry or for immunoblotting.

Morphological studies

The cellular location of some junctional and cytoskeletal proteins was determined by a standard indirect immunofluorescence technique in cryosections of isolated neonatal islets and of whole pancreas of adult rats (Collares-Buzato *et al.* 2001). Briefly, a pool of isolated islets (~1000–2000 islets) was fixed in 2% paraformaldehyde plus 10% saccharose (in 0.01 M phosphate-buffered saline, PBS, pH 7.4) for 30 min and included in gelatin solutions of increasing concentration (5, 10 and 25%) at 37 °C. The gelatin blocks or unfixed pancreas were frozen in *n*-hexane with liquid nitrogen. Cryostat sections of pancreas were picked up on poly-L-lysine-coated glass slides and fixed for 10 min with 2% paraformaldehyde. Islet and pancreas sections were then treated with 0.1% Triton X-100 (in PBS) and incubated with primary antibody. The following primary antibodies were used at the indicated dilutions (in PBS, pH 7.4): rabbit anti-occludin (dilution 1 : 50) (Zymed), rabbit anti-ZO-1 (dilution 1 : 50) (Zymed), mouse anti-β-catenin (dilution 1 : 50) (Zymed), rabbit anti-α-catenin (dilution 1 : 50) (Sigma), mouse anti-α-actinin (dilution 1 : 50) (Upstate) and mouse anti-vinculin (dilution 1 : 50) (Sigma). Following incubation with the specific secondary antibody conjugated to fluorescein (dilution 1 : 75), the

sections were mounted in a commercial antifading agent (Vectashield, Vector Laboratories, Burlingame, CA, USA). Double immunolabeling of β-catenin or ZO-1 and insulin was also done described above but with two additional incubation steps with guinea pig anti-insulin (dilution 1 : 500) (Dako) for 1 h followed by another 1 h incubation with the specific secondary antibody conjugated to rodhamine (dilution 1 : 100). Cell labeling was visualized by confocal laser scanning microscopy (CLSM; BioRad MRC 1024UV; Bio-Rad Laboratories, Hercules, CA, USA) using an inverted fluorescence microscope (Nikon). Stainings were absent from all the negative control tissue sections (pancreas) in which the primary antibody tested (i.e. all of one listed above) was replaced by 3% horse serum in PBS.

The cytochemistry for F-actin was done in cryosections of paraformaldehyde-fixed and Triton-permeabilized pancreas and isolated islets by incubating them with TRITC-labeled phalloidin (diluted 1 : 50, in PBS, pH 7.4) at 37 °C, in the dark. All cell labelings were visualized by CLSM.

To allow comparison of the degree of fluorescence among the experimental groups, the immunostaining and microscopic examination of islets cultured for different time periods were done simultaneously, with the same antibody aliquot, in the same experimental session using the same CLSM sensitivity. The observations were confirmed in at least three independent sets of experiments.

Western blotting

Immunoblotting to detect the expression of junctional proteins in islet cells was done as previously described (Collares-Buzato *et al.* 2001). Briefly, a pool of at least 1000 islets from each experimental group was homogenized by sonication in an antiprotease cocktail (10 mM imidazole, pH 8.0, 4 mM EDTA, 1 mM EGTA, 0.5 μg of pepstatin A/ml, 200 KIU of aprotinin/ml, 2.5 μg of leupeptin/ml, 30 μg of trypsin inhibitor/ml, 200 μM DL-dithiothreitol, DTT, and 200 μM phenyl methyl sulfonyl fluoride, PMSF). The sonicate was centrifuged at 3000 × *g* for 10 min (4 °C), and the supernatant was collected and its total protein content determined using a DC protein assay kit (Bio-Rad). Samples of each experimental group containing 70 μg of total protein were fractionated by electrophoresis in 6.5% (for ZO-1 and occludin) or 8% (for β-, α-catenins and vinculin) polyacrylamide gels and, the proteins then electrotransferred to nitrocellulose membranes (Bio-Rad). The membranes were stained with Ponceau S solution (Sigma) to check efficiency of transfer and only those membranes in which all of the lanes had an identical color intensity, indicating identical protein loading, were used for immunoblotting. The membranes were initially incubated with the primary antibody (the same as used in immunocytochemistry) (dilution 1 : 500) followed by incubation with I^{125}-labeled protein A (dilution 1 : 1000) (Amersham). In case of β-catenin, an additional incubation with a rabbit anti-mouse IgG (dilution 1 : 1500) was done between the primary antibody and the protein A incubation steps. Radiolabeled protein bound to the antibodies was detected by autoradiography. Band intensities were quantified by optical densitometry of the developed autoradiogram using the Scion image analysis software, Beta 4.02 for Windows.

Insulin secretion

After culture, the islets were gently detached from the plates under a dissecting microscope. Groups of 8–10 clean islets from the different experimental groups were placed in the wells of 24-well plates and incubated for 2 h at 37 °C with 1 ml of bicarbonate-buffered Krebs solution (pH 7.4) (composition in mM: NaCl 115, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 24 and glucose 10, supplemented with 3 mg of bovine serum albumin/ml and equilibrated with a mixture of 95% O_2–5% CO_2). After a 2 h incubation, aliquots of the supernatant were taken and stored at −20 °C. The insulin content of these samples was determined by radioimmunoassay and was expressed as nanograms per islet/h (Collares-Buzato *et al.* 2001).

Statistical analysis

All numerical results were expressed as the means ± standard error (SE). For multiple comparisons, the statistical significance was assessed by ANOVA followed by the Bonferroni test. The significance level was set at $P < 0.05$.

Results

In agreement with previous reports (Boschero *et al.* 1988, 1993, Crepaldi-Alves *et al.* 1997), culture of neonatal rat islets induces significant increase in insulin release into the culture medium. Islets cultured for 3 days (0.46 ± 0.06 ($n = 17$ wells; 8–10 islets per well) ng insulin/islet/h; $P < 0.05$) or 7 days (1.030 ± 0.17 ($n = 16$ wells; 8–10 islets per well) ng insulin/islet/h; $P < 0.001$) showed an approximately 2-fold and 3-fold increase in insulin release compared to 24 h-cultured islets (0.26 ± 0.02 ($n = 17$ wells; 8–10 islets per well) ng insulin/islet/h).

As shown in Figures 1 and 2, neonatal rat islets cultured for only 24 h expressed a relatively low amount of tight junctional (occludin and ZO-1) and adherens junctional (α- and β-catenins) proteins, as seen in

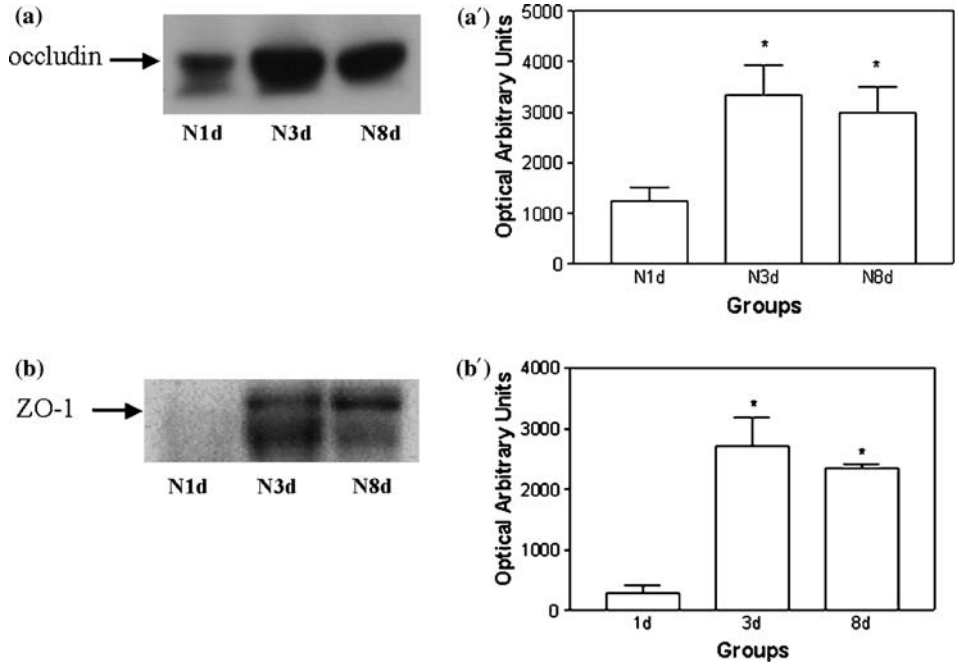

Figure 1. Increase in the expression of the tight junctional proteins ZO-1 and occludin in neonatal pancreatic islets following tissue culture. The levels of both junctional proteins were assessed by Western blotting of islet sonicates. Note that N3d (lane 2) and N8d (lane 3) showed a significantly higher expression of these junctional proteins than N1d (lane 1) (in a and b). The antibody against occludin recognized a broad band between 41.8 and 71 kDa corresponding to the occludin protein (~65 kDa) whereas the antibody against ZO-1 recognized two bands just above 202 kDa corresponding to the two isoforms α^+ and α^- isoforms of this protein. The densitometric values for each experimental group shown in (a′) and (b′) are the mean + SE of five and three independent experiments, respectively. In (b′), the densitometric data represent both ZO-1 isoforms. Legends: N1d, neonatal islets cultured for 24 h; N3d, neonatal islets cultured for 3 days; N8d, neonatal islets cultured for 8 days. $*P < 0.05$ in comparison with the N1d.

Western blots. However, after 3 or 8 days in culture, there was a significantly greater expression of all of these junctional proteins; a maximum level of expression was obtained after 3 days in culture. In islets cultured for 3 days there was an approximately 3-fold and 10-fold increase in the expression of the TJ-associated occludin and ZO-1, respectively, (Figure 1) and a 6-fold and 3-fold increase in α- and β-catenin expression, respectively (Figure 2). However, there were no changes in the expression of the cytoskeletal protein, vinculin, in islet homogenates after culturing (Figure 2c, c′).

These findings obtained by immunoblotting were confirmed by immunocytochemical analysis. As shown in Figures 3 and 4, pancreatic islets cultured for only 24 h contained endocrine cells that stained faintly for ZO-1 (Figure 3b) and β-catenin (Figure 4c) distributed throughout the cytoplasm. A similar pattern of staining for α-catenin was observed in islet sections, but was greater than that observed for the other junctional proteins (Figure 4a). In islets cultured for 3 days, there was a dramatic increase in the cell content of the junctional proteins ZO-1 and β-catenin, and a moderate increase associated with a translocation of α-catenin to the junctional region as observed by indirect immunofluorescent labeling. In addition, the stainings for all the junctional proteins were confined mainly to the regions of cell–cell contact (Figures 3 and 4). There were no changes in the expression or cell distribution of the cytoskeletal proteins vinculin and α-actinin in neonatal islets after culturing (Figure 5). Immunolabeling for these proteins revealed a relatively faint cytoplasmic distribution in islet cells, regardless of the duration of culture. Figure 6 shows that neonatal islets cultured for 3 days had a higher content of F-actin associated with the junctional region of the membrane compared with islets cultured for only 24 h.

To confirm that these changes in the expression and distribution of IJ-associated proteins occurred mainly in B-cells and not in undifferentiated islet cells or non-endocrine cells, dual immunolabeling for β-catenin and insulin was done in neonatal islets cultured for 7 days. As shown in Figure 7, there was a significant increase in the junctional content of β-catenin in insulin-secreting B-cells after culturing. Non-B-cells were also immunoreactive for this junctional protein (Figure 7).

β-Catenin and ZO-1 were also expressed by cells in islets of Langerhans of adult rats *in situ* (Figure 8). Both B- and non-B-cells showed a similar junctional localization of both proteins. Cytochemistry with phalloidin revealed that the endocrine cells of the adult pancreas had actin microfilaments that were concentrated mainly around the IJs.

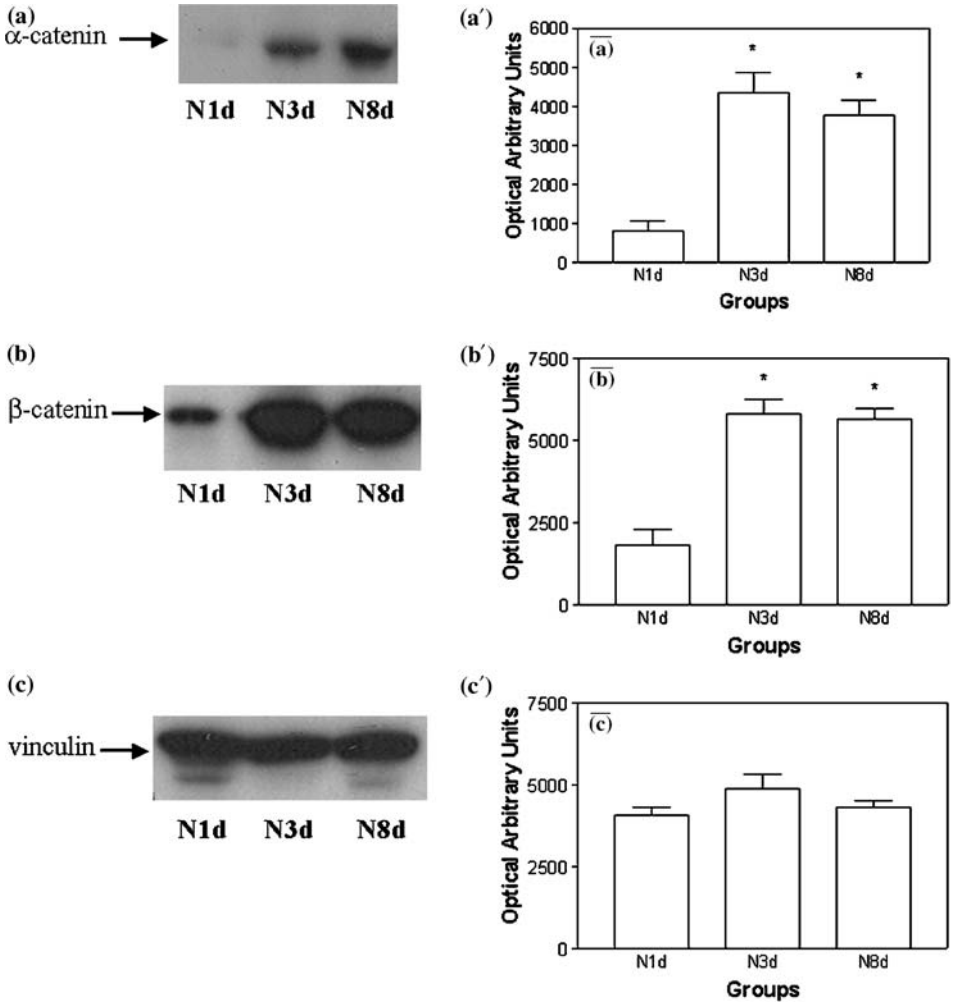

Figure 2. Increase in the expression of the adherens junction-associated proteins α- and β-catenins, but not of vinculin, in neonatal pancreatic islets following tissue culture. The levels of these junctional proteins were assessed by Western blotting of islet sonicates. Note that N3d (lane 2) and N8d (lane 3) showed a significantly higher expression of both catenins than N1d (lane 1) (in a and b), recognized by the primary antibodies as single band between 71 and 133 kDa corresponding to (a) α- (102 kDa) and (b) β-catenin (92 kDa). There were no differences after islet culturing in the expression of the cytoskeletal protein vinculin, that appeared as a single band below 133 kDa in all of the immunoblots (c, c′). The densitometric values for each experimental group shown in (a′–c′) are expressed as the mean + SE of at least three independent experiments. Legends: N1d, neonatal islets cultured for 24 h; N3d, neonatal islets cultured for 3 days; N8d, neonatal islets cultured for 8 days. $^*P < 0.05$ in comparison with the N1d.

Discussion

Insulin secretion is a complex, multistep process regulated by metabolic and neurohormonal signals, as well as by direct cell–cell interactions among islet cells (Halban *et al.* 1982, Bosco *et al.* 1989, Pipelleers *et al.* 1994). Intercellular contacts are established through membrane specializations such as adherens junctions, desmosomes, gap and tight junctions. Each of these forms of cell contact has been ultrastructurally identified in islet cells, but their functional significance and biochemical composition are not yet fully understood (Orci *et al.* 1975, Bendayan 1981, Yamamoto & Kataoka 1984, 1988). To our knowledge, the present study is the first study to assess the biochemical features of these IJs in the endocrine pancreas. Our results indicate that the expression of these junctional proteins can be regulated in a specific manner (since cytoskeleton-associated proteins were not affected) in neonatal islets following extended culture. These findings indicate that IJs are dynamically regulated structures in islet cells and, by analogy to their role in other tissues, they may influence the differentiation, growth and secretory processes in the endocrine pancreas. In support of this idea, the culture-induced increase in the expression of junctional proteins occurred in parallel with an improvement in the insulin secretory response of cultured neonatal islets. It is well documented that fetal and neonatal rat islets exhibit a reduced secretory response to glucose when compared with adult islets (Masaki *et al.* 1987, Boschero *et al.* 1988, 1993). In

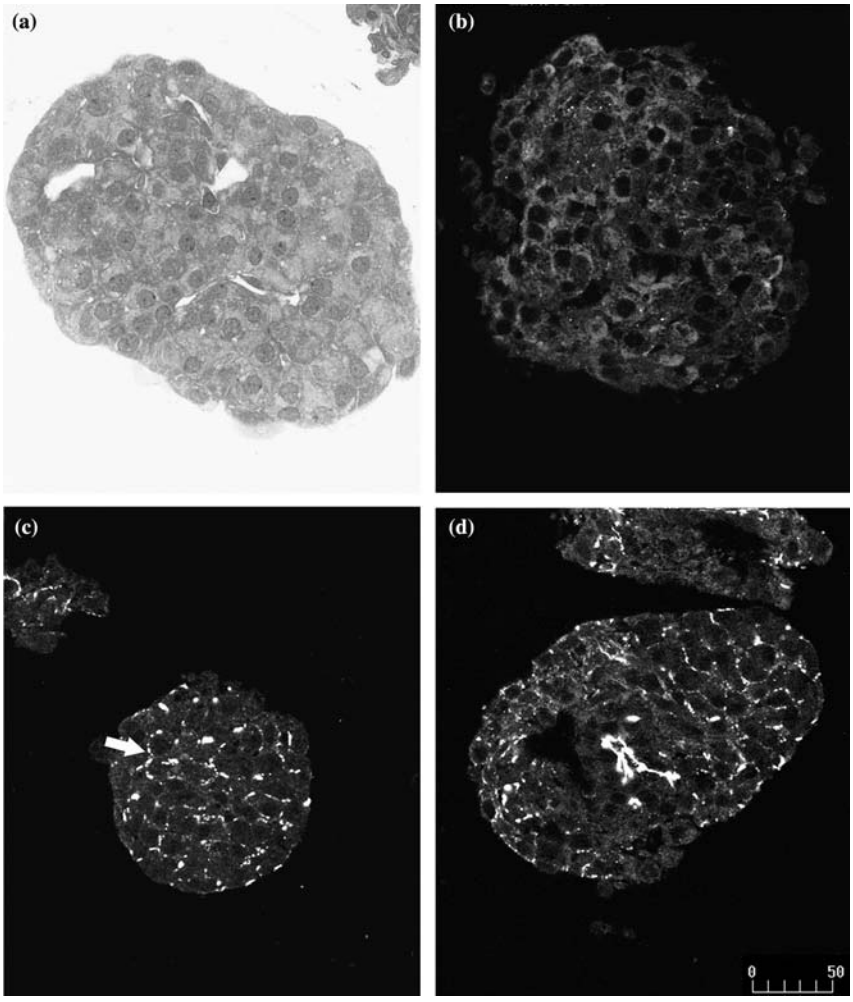

Figure 3. Increased junctional content of ZO-1 in islet cells following tissue culture: (b–d) 'En face' (X–Z) confocal images showing indirect immunoflorescence staining of ZO-1 in neonatal islets cultured for 24 h (b) or 3 days (c, d). The images were obtained using the same sensitivity of the confocal laser scanning microscope. ZO-1 was seen as bright, discontinuous labeling in regions of cell–cell contact in 3-day cultures of neonatal islets (c, arrow) whereas islets cultured for only 24 h showed a faint, diffuse labeling for this protein (b). The bright, linear labeling in (d) may correspond to an islet capillary. In (a), the optical micrograph shows the appearance of an islet after 3 days in culture. For this analysis, a pellet of cultured islets was fixed in 2% paraformaldehyde, embedded in historesin and sections (5 μm thick) were stained with blue toluidine. Scale bar, μm.

accordance with our work, numerous studies have shown that fetal and neonatal islets, when cultured for a certain period of time, display an increased insulin response to suprastimulatory concentrations of glucose as well as to other secretagogues (Shimizu *et al*. 1983, Dudek *et al*. 1984, Freinkel *et al*. 1984, Masaki *et al*. 1987, Boschero *et al*. 1993, Yderstraede & Flindt-Egebak 1995, Collares-Buzato *et al*. 2001). Such process of *in vitro* maturation is even enhanced by exposure to prolactin (Boschero *et al*. 1993, Crepaldi *et al*. 1997, Crepaldi-Alves *et al*. 1997, Collares-Buzato *et al*. 2001). It has been suggested that the gradual increase in insulin secretory capacity of islets and fetal pancreata *in vitro* can not only be explained by islet hyperplasia but involves the maturation of critical steps of stimulus-secretion coupling process in the B-cell (Dudek *et al*. 1984, Freinkel *et al*. 1984, Boschero *et al*. 1993, Yderstraede & Flindt-Egebak 1995, Crepaldi *et al*. 1997, Crepaldi-Alves *et al*. 1997). The culture of fetal or neonatal islets, therefore, can constitute a valuable tool for investigating the role of the IJ-mediated islet cell aggregation upon the secretory function of the endocrine pancreas.

Tight junctions (TJs) have been well studied in transporting epithelia, where they function as a regulated, paracellular route-limiting permeability barrier (Collares-Buzato *et al*. 1994a, b, Stevenson & Keon 1998, Zahraoui *et al*. 2000). In transmission electron micrographs of thin sections, TJs appear as a single or a series of discrete sites of apparent fusion ('kisses'), involving the outer leaflet of the plasma membrane of adjacent cells (Faquhar & Palade 1963, Stevenson & Keon 1998). Freeze–fracture images of

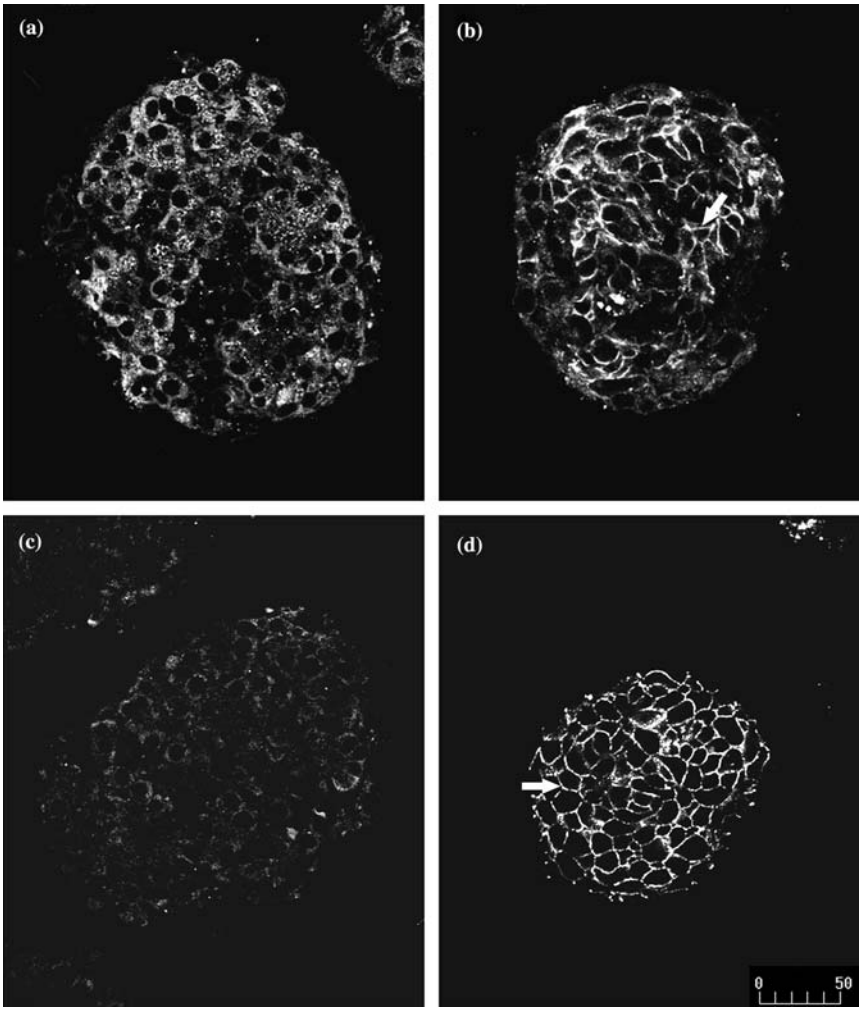

Figure 4. Increased junctional content of α- (a,b) and β-catenins (c,d) in islet cells following tissue culture: (a–d) 'En face' (X–Z) confocal images showing indirect immunoflorescence staining of α- and β-catenins in neonatal islets cultured for 24 h (a, c) or 3 days (b, d). The images were obtained using the same sensitivity of the confocal laser scanning microscope. Both catenins appeared as a bright ring pattern of labeling at the cell–cell contact sites in neonatal islets cultured for 3 days (arrows in b and d) while those cultured for only 24 h showed a faint, diffuse labeling for both proteins (a, c). Scale bar, μm.

TJs in epithelia show a complex network of anastomosing strands and complementary grooves that represent the 'kiss' sites identified in thin sections (Stevenson & Keon 1998). Transmembrane integral proteins (occludin and claudins) form the epithelial TJ strands and bind to a complex of cytoplasmic submembranous proteins (ZO-1, ZO-2, ZO-3, cingulin, 7H6 antigen and symplekin) (Stevenson and Keon 1998, Zahraoui 2000). These cytoplasmic proteins appear to organize the occludin and claudins within the TJ site and couple them to actin microfilaments. Interactions between TJ-associated proteins and the cytoskeleton play a pivotal role in the regulation of epithelial paracellular permeability (Collares-Buzato *et al*. 1994a, b, Zahraoui 2000).

In contrast to epithelia, the functional and biochemical features of the TJs in non-transporting epithelia have been largely ignored. In the endocrine pancreas, TJs between endocrine cells have been identified by transmission electron microscopy and freeze-fracturing studies. (Orci *et al*. 1975, Bendayan 1981, Yamamoto & Kataoka 1984, 1988). Unlike the belt-like feature of TJs in transporting epithelia, these junctions appear as short anastomosing strands frequently associated with small clusters of gap junctional particles in islet cells (Yamamoto & Kataoka 1984, 1988). The number of focal TJs (or macula occludens) increases in isolated islets under experimental conditions that enhance the insulin secretory activity, i.e. after exposure to pronase and glucose (Orci *et al*. 1973, Semino *et al*. 1987). In agreement with these data, we have shown here that neonatal isolated islets express the TJ-associated proteins occludin and ZO-1 and that both junctional proteins were upregulated *in vitro*. Immunoblots for ZO-1 revealed two immunoreactive bands above 200 kDa that may represent the α^+ and α^--isoforms of this pro-

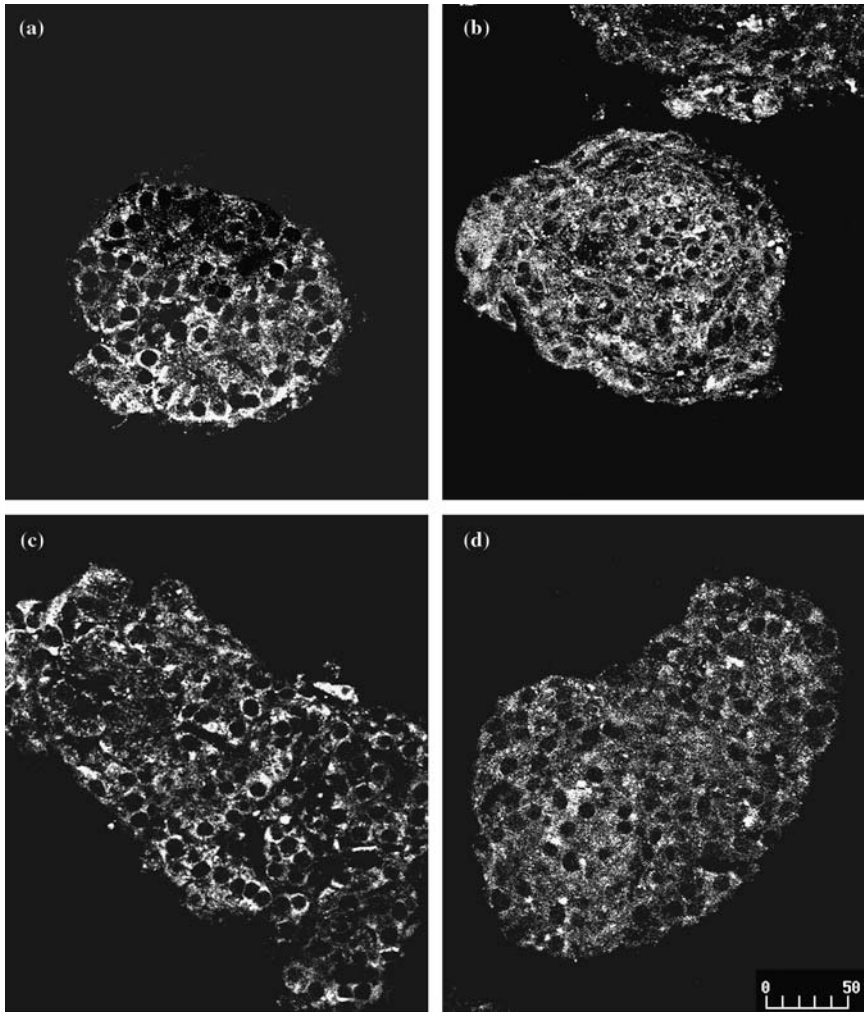

Figure 5. Unaltered immunoreactivity for the cytoskeleton-associated proteins vinculin and α-actinin in cultured islets: (a–d) 'En face' (X–Z) confocal images showing indirect immunoflorescence staining of vinculin and α-actinin in neonatal islets cultured for 24 h (a, c) or 3 days (b, d). The images were obtained using the same sensitivity of the confocal laser scanning microscope. Note that vinculin (a, b) and α-actinin (c, d) showed a diffuse, cytoplasmic labeling in both 24 h (a, c) and 3 days (b, d) cultures of neonatal islets. Scale bar, μm.

tein since the antibody used recognized both ZO-1 isoforms (manufacturer's data). Interestingly, the presence of the α^{-}-isoform of ZO-1 has been associated with cells displaying structurally dynamic TJs, such as podocytes, Sertoli cells and mammary gland cells (Balda & Anderson 1993, Stelwagen *et al.* 1999). In these cells, TJs can rapidly open and reseal.

ZO-1 appeared as discontinuous lines in the cell–cell contact region and resembled the macula occludens seen ultrastructurally between endocrine pancreatic cells. The finding that ZO-1 was also present in the endocrine pancreas of adult rats implies a possible role for this TJ-associated protein in this organ *in situ*. However, this result differs from other studies that found no evidence for TJs in the normal endocrine pancreas of certain animal species, including rats and humans (In't Veld *et al.* 1984, Yamamoto & Kataoka 1984). According to these studies, rather than being involved in normal islet cell function, TJs may provide an adaptative structure intended to protect the islet microdomain against sudden perturbations such as occuring during islet isolation. We have no explanation for the discrepancy of these studies and our data. However, it should be mentioned that, although ZO-1 is an ubiquitous protein in TJ, in the absence of this junction this protein may associate with adheren-type junctions in other cells (Howarth *et al.* 1992, Itoh *et al.* 1993, 1997). The functional importance of TJs in the endocrine pancreas *in situ* requires further investigation.

Cultured neonatal islets also express the adherens-associated proteins α- and β- catenins. Catenins are cytoplasmic proteins that connect cadherin adhesion molecules to actin filaments (Geiger & Ayalon 1992, Geiger *et al.* 1995, Harington & Syrigos 2000). Perturbation of the cadherin–catenin interaction or decreased expression of either of the components of this complex invariably leads to reduced cell–cell adhesion in several cell types (Collares-Buzato *et al.*, 1998a, b, Harington & Syrigos

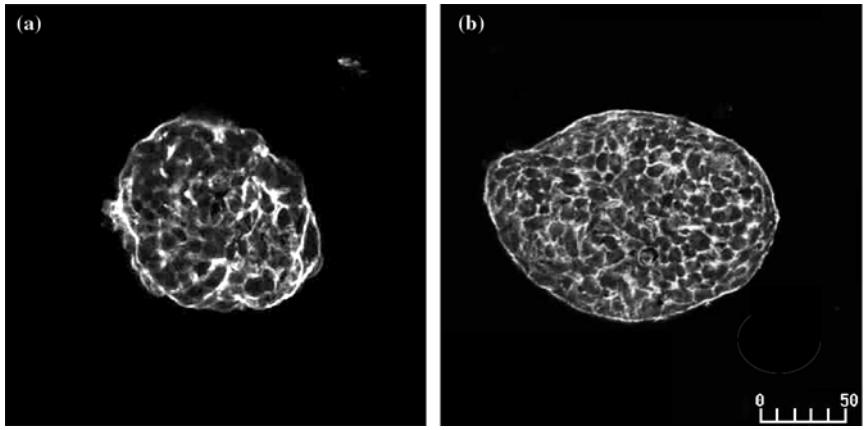

Figure 6. F-actin distribution in islets cultured for 24 h or 3 days: 'En face' (X–Z) confocal images showing cytochemistry for F-actin using TRITC-phalloidin in neonatal islets cultured for 24 h (a) or 3 days (b). The images were obtained using the same sensitivity of the confocal laser scanning microscope. Note the higher junctional labeling for F-actin in neonatal islets cultured for 3 days compared to those for only 24 h. Scale bar, μm.

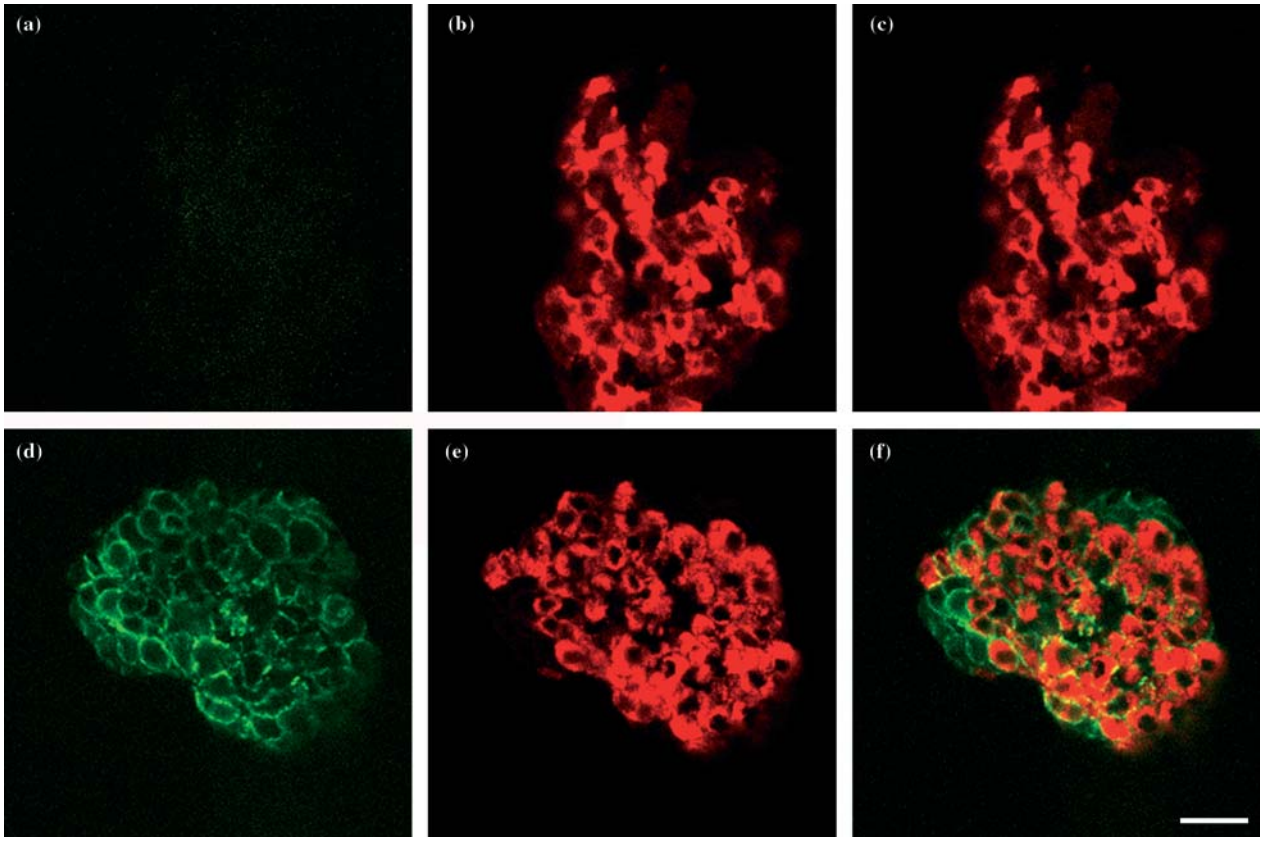

Figure 7. Dual immunolabeling for β-catenin and insulin in islets cultured for 24 h or 3 days: (a–f) 'En face' (X–Z) confocal images showing dual immunoflorescence staining of β-catenin (a, d) and insulin (b, e) in neonatal islets cultured for 24 h (a–c) or 3 days (d–f). All images were obtained using the same sensitivity of the confocal laser scanning microscope. Note that insulin secreting B-cells in 24 h cultures showed no immunoreaction for β-catenin at this level of CLSM sensitivity (a–c), while in islets cultured for 7 days, B-cells displayed a bright ring pattern of labeling at cell–cell contact sites (d–f). In addition, non-B-cells, in 3 day-cultured islet, also show junctional immunolabeling for β-catenin (f). Scale bar, μm.

2000, Ling *et al*. 2001, Liliem *et al*. 2002). Cadherins are cell surface glycoproteins responsible for Ca^{2+}-dependent, homophilic intercellular adhesion. The cadherin–catenin also plays a pivotal role in cell migration, differentiation, cell–cell recognition, cell fate and death (Geiger & Ayalon 1992, Geiger *et al*. 1995, Harington & Syrigos 2000). Islet cells express the E-cadherin subtype, as well as N-CAM, a Ca^{2+}-independent cell adhesion

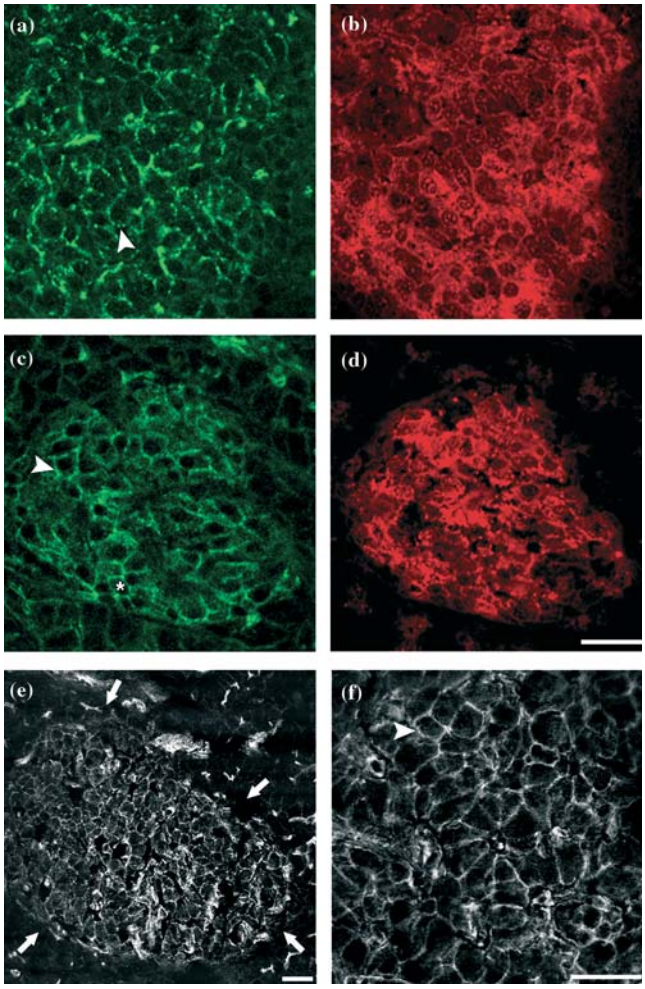

Figure 8. Expression and distribution of ZO-1, β-catenin and F-actin in the endocrine pancreas of adult rat: (a–d) 'En face' (X–Z) confocal images showing dual immunoflorescence staining of ZO-1 (a), β-catenin (c) and insulin (b, d) in islets of Langerhans. (e, f) 'En face' (X–Z) confocal images of the endocrine pancreas of rats following cytochemistry with TRITC-conjugated phalloidin to detect F-actin. Note that insulin secreting B-cells stained for ZO-1 and β-catenin in the intercellular junction region (arrowheads in a and c). In addition, cells of the endocrine pancreas (in e, arrows indicate the islet periphery) show a concentration of F-actin in the cell–cell contact region (arrowhead in (f)). Asterisk in (c) indicates a non-B-cell. Scale bars, 30 μm.

molecule (CAM) belonging to the immunoglobulin superfamily (Moller *et al*. 1992, Bernard-Kargar *et al*. 2001). Non-B-cells express significantly higher levels of N-CAM compared to B-cells, whereas E-cadherin is identically expressed by both cell types. The differential expression of N-CAM has been accounted for the typical topographical arrangement of cells within the islet characterized by a core of insulin-secreting B-cells surrounded by non-B-cells secreting glucagon, somatostatin and polypeptide P (Moller *et al*. 1992, Bernard-Kargar *et al*. 2001).

Exposure to tumor necrosis factor or antibodies against E-cadherin results in an abnormal islet architecture and a decreased cell adhesion, respectively, which are associated with a decrease in insulin release (Cirulli *et al*. 1993, Yamagata *et al*. 2002). These findings reinforce the idea that CAM-mediated cell–cell contact is crucial for a proper secretory response by the endocrine pancreas. In agreement with this idea, we found an increase in the expression of catenins and in the junctional content of F-actin in neonatal islets cultured for more than 3 days. In addition, the immunolocalization of catenins at the intercellular region in the adult endocrine pancreas and cultured neonatal islets agreed with the islet expression of E-cadherin shown by others. Catenins appear to be expressed similarly in both cell types, B- and non-B-cells, as revealed by dual immunolabeling for β-catenin and insulin.

In conclusion, endocrine pancreatic cells express several junctional proteins (occludin, ZO-1, and α- and β-catenins) that are upregulated following differentiation of the endocrine pancreas *in vitro*. Culturing neonatal islets also increased the junctional content of F-actin, but did not alter the expression of cytoskeleton-associated proteins, such as vinculin and α-actinin. ZO-1 and β-catenin were observed in the endocrine pancreas of adult rats, indicating that these junctional proteins were also expressed *in situ*. Further studies *in vitro* and *in situ* on the IJs and their constitutive proteins will lead to a better understanding of the functional role of these important structures in B-cells.

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