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Cadherin Engagement Protects Human β-Cells from Apoptosis

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The aim of this study was to assess the expression of different types of cadherins in human islets and their role in human β-cell apoptosis. Expression of E-, N-, and P-cadherins was studied by immunofluorescence on pancreas sections and islet cells, and by Western blotting on protein extracts of isolated islets and islet cells. The effects of specific cadherins on cell adhesion and apoptosis were studied using chimeric proteins containing functional E-, N-, or P-cadherin ectodomains fused to Fc fragment of Ig (E-cad/Fc, N-cad/Fc, and P-cad/Fc) and immobilized on glass substrate. β-Cells were identified by immunofluorescence for insulin and apoptotic cells by terminal deoxynucleotide transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick-end labeling. By immunofluorescence, we showed that E- and N-, and not P-, cadherins were expressed at the surface of islet cells. By triple staining, we showed that E-cadherin was expressed at similar extent in β- and α-cells, whereas N-cadherin was preferentially expressed in β-cells. These results were confirmed by Western blot analysis using protein extracts from fluorescence-activated cell sorting-sorted β- and non-β-cells. Adhesion tests showed that the affinity of islet cells for E-cad/Fc and N-cad/Fc and not for P-cad/Fc was increased compared with control. By terminal deoxynucleotide transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick-end labeling, we showed that the percentage of apoptotic cells was lower in aggregated β-cells compared with single β-cells and that attachment to E-cad/Fc and N-cad/Fc and not to P-cad/Fc decreased apoptosis of single β-cells compared with control. Our results show that at least E- and N-cadherins are expressed at the surface of human β-cells and that these adhesion molecules are involved in the maintenance of β-cell viability. **(*Endocrinology* 152: 4601–4609, 2011)**

In pancreatic islets, apoptosis has been extensively studied, because this form of cell death is associated with diabetes (1, 2) and is involved in the rapid loss of β-cell mass after islet isolation and consequently in the limited success of islet transplantation (1, 3). Hyperglycemia, hyperlipidemia, oxidative stress, local production of cytokines, and other adverse factors produced by inflammatory cells contribute to β-cell apoptosis (2). Furthermore, after islet transplantation, β-cells are exposed to adverse effects of immunosuppressive drugs and allogeneic immune reactions (4). Deciphering the mechanisms of β-cell apoptosis is important for the comprehension and prevention of diabetes and the success of the treatment of this disease by islet transplantation. Numerous strategies have been explored to inhibit β-cell apoptosis (2, 3), and research has mainly focused on the modulation of production of effectors known to affect apoptosis, such as IL-1, interferon (IFN)γ, nitric oxide, TNFα, and Fas ligand. On the other hand, anoikis is a form of apoptosis that is induced by the disruption of cell anchorage, involving cell-to-extracellular matrix (5) and cell-to-cell interactions (6). Integrin-extracellular matrix interaction has been shown to maintain low level of apoptosis in β-cells *in vitro* (7–9), and recently, it has been reported that cell-to-cell coupling

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Abbreviations: FACS, Fluorescence-activated cell sorting; IFN, interferon; NCAM, neural cell adhesion molecule; T1D, type 1 diabetes; TUNEL, terminal deoxynucleotide transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick-end labeling.

mediated by connexin36 protected cytokine-induced apoptosis in β-cells (10). It is still undetermined whether cell adhesion molecules by a coupling-independent mechanism also play a role in apoptosis of β-cells. Cadherins are a family of calcium-dependent adhesion molecules widely expressed in epithelial cell types, including pancreatic islet cells. In addition to provide structure and integrity to epithelia, cadherins initiate cell signaling through activation of intracellular pathways involving protein phosphorylation and are able to modulate cell differentiation, polarity, replication, and viability. These effects of cadherins require cadherin-cadherin engagement, which serves as signal receptor activation linking cadherin to catenin/cytoskeleton complex and others signaling pathways (6). In many systems, mostly related to cancer, it has been shown that there is a correlation between modification of specific cadherin expression, proliferation, and apoptosis (11–16).

Whether cadherins play a role in islet cell viability has not yet been determined. In islets, specific cadherins are known to control, at selected developmental stages, the adhesion of endocrine cells and characteristic organization of different cell types within these endocrine units (17–19). In addition, cadherins have been reported to be expressed at different levels in β-cells, in which their expression correlates with a distinct insulin secretion (20). Also, RNA interference-mediated silencing of specific cadherins in aggregated MIN6 insulinoma cells resulted in a decreased glucose-stimulated insulin secretion (21), and exposure of MIN6 cells to an anticadherin antibody blocked the elevation of intracellular calcium concentration in response to glucose stimulation (18). In this work, we aimed to characterize expression of different cadherins in human islets and to assess whether apoptosis of β-cells could be affected by cadherin expression and engagement. This objective was challenging, mainly due to the difficulty to distinguish between signals that were activated directly by cadherin ligation and signals activated by other types of cell-to-cell interactions, such as gap junction communication. To surpass this complication, we used a strategy previously described by Yap and co-workers (22), consisting in the activation of E-cadherin on single cells (deprived of intercellular contacts) by its ligation to specific cadherin peptides attached to an inert substrate.

Materials and Methods

Islet cell isolation and cell culture

Human pancreata were obtained from braindead multiorgan donors. In some occasion, small pancreatic samples were taken for histological analysis before islet isolation. Samples were embedded into Tissue-Tek Optimal Cutting Temperature and snap frozen in liquid nitrogen for subsequent histological analyses. The use of human islets for research was approved by our local institutional ethical committee. Islet isolation was performed with a technique modified from the semiautomated method developed as in Ref. 23 and by Ricordi *et al.* (24). Pancreata were perfused with cold enzyme solution and incubated at 37 C in a digestion chamber. Islets were then purified in continuous Biocoll (Biochrom KG, Berlin, Germany) gradients using a COBE 2991 cell processor (Cobe, Lakewood, CO). Islets were cultured in CMRL 1066-medium, containing 5.6 mmol/liter glucose and supplemented with penicillin, streptomycin, glutamine, HEPES, and 10% fetal calf serum (hereafter referred to as complete CMRL). The use of human islets for research was approved by our local institutional ethical committee. After purification, islets were cultured overnight at 37 C and, at 25 C thereafter, in CMRL medium. To dissociate islets into single cells, 1000–5000 islet equivalent were rinsed twice with 10 ml of PBS, resuspended in 1 ml of Accutase (Innovative Cell Technologies, San Diego, CA), and incubated at 37 C with gentle pipetting every 30 sec. From 5 min onwards, complete cell dissociation was checked by microscopic observation of 10-μl aliquots sampled every min. When dissociation was considered to be complete (usually between 7 and 10 min), cells were diluted with 10 ml of cold complete CMRL. At this time, 95–98% of islet cells were single. When needed, islet cells were sorted in β-cell and non-β-cell fractions by fluorescence-activated cell sorting (FACS) as previously described (25). Aliquots of 10^5 islet cells or FACS-sorted islet cells were incubated at the indicated time at 37 C in 10-cm-diameter nonadherent Petri dishes containing 10 ml of complete CMRL, before using for adhesion assay, apoptosis analysis, Western blotting, or immunofluorescence. A human keratinocyte cell line (HaCaT) was used as control for P-cadherin expression. HaCaT cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin, and streptomycin, at 37 C in 5% CO_2. HaCaT cells were grown to confluence before protein extraction. For immunofluorescence, HaCaT cells were growth to confluence on coverslips.

Immunofluorescence

Frozen pancreas samples were sectioned (5 μm) using a Leica CM1950 Cryostat (Leica Microsystems, Bannockburn, IL) and affixed to Superfrost Plus slides. Islet cells were injected into Cunningham chambers previously coated with 0.1 mg/ml poly-L-lysine and incubated 1 h at 37 C. For immunostaining, islet cells and pancreas sections were fixed in 10% methanol-free formalin for 20 min. Islet cells were permeabilized 10 min with 0.1% Triton X-100 in PBS. Islet cells and pancreas sections were rinsed in PBS, incubated in 0.5% BSA in PBS for 10 min, and then exposed 2 h to a combination of primary antibodies as indicated in *Results*. Primary antibodies against cadherins were a mouse anti-E-cadherin purchased from Zymed Laboratories (South San Francisco, CA) diluted 1:80, a rabbit anti-E-cadherin from Cell Signaling Technology (Danvers, MA) diluted 1:40, a mouse anti-N-cadherin from BD Transduction Laboratories (San Diego, CA) diluted 1:50, a mouse anti-N-cadherin from Sigma (St. Louis, MO) diluted 1:50, and a mouse anti-P-cadherin from BD Transduction Laboratories diluted 1:50. The specificity of these antibodies was tested on different organ sections. For instance, cell-to-cell contacts of all epithelial cells present on liver or pancreas sections were labeled, whereas smooth muscle cells in ar-

teries or cardiomyocytes in human heart were not labeled by anti-E-cadherin antibodies. On the contrary, the N-cadherin antibody stained smooth muscle cells; and in heart, staining was, as expected, localized in the intercalated discs at cell-to-cell junctions. The other antibodies used were a rabbit anti-β-catenin from Sigma diluted 1:500, a guinea pig antiinsulin diluted 1:500, a rabbit antiglucagon diluted 1:100, a rabbit antisomatostatin diluted 1:400, a rabbit anti-pancreatic polypeptide diluted 1:400 from Dako (Baar, Switzerland), and a mouse antiglucagon from Sigma diluted 1:4000. After rinsing in PBS, islet cells and pancreas sections were exposed to an adequate combination of fluorescence labeled secondary antibodies for 1 h. Fluorescence-labeled secondary antibodies were purchased from Jackson ImmunoResearch (Rheinfelden, Switzerland). Islet cells and pancreas sections were rinsed in PBS and coverslipped before to be observed and photographed using an Axioskop microscope (Zeiss, Feldbach, Switzerland) equipped with a color charge-coupled device camera, UV illumination, and filters for blue, red, and green fluorescences. Microscopic fields of islets acquired from pancreas section immunostained for E- or N-cadherin and insulin and glucagon were analyzed for pixel intensity of cadherin-specific immunoreactivity using the offline MetaMorph imaging software for microscopy (Universal Imaging Corp., West Chester, PA). Pixel intensity was quantified separately in manually selected regions stained for glucagon and insulin. Pixel intensity of background on each microscopic field was subtracted.

Western blotting

Protein extracts were prepared by homogenization of islets, islet cells, and HaCaT cells in a 0.1 M Tris-HCl buffer (pH 7.4), supplemented with 5 mM EDTA, 5% sodium dodecyl sulfate, and protease inhibitors cocktail (Roche Diagnostic, Mannheim, Germany), followed by sonication. Protein content was measured using a protein assay kit (Bio-Rad Laboratories, Glattbrugg, Switzerland) and aliquots stored at −20 C before to be processed by Western blotting. Proteins prepared from islets, and islet cells were separated by electrophoresis in 7.5% polyacrylamide gels. Proteins were transferred electrically onto polyvinylidene fluoride membranes (Millipore, Billerica, MA) using a constant current of 450 mA for 75 min. Polyvinylidene fluoride membranes were saturated with 5% dried milk and 0.1% Tween-20 in Tris-HCl buffer (pH 7.4) containing 150 mM NaCl (Tris-buffered saline-Tween) for 1 h and then exposed overnight at 4 C to anti-E-cadherin antibody (Cell Signaling Technology) diluted 1:1000, anti-N-cadherin antibody (BD Transduction Laboratories) diluted 1:2500 or anti-P-cadherin antibody (BD Transduction Laboratories) diluted 1:800. After three washes with Tris-buffered saline-Tween, membranes were incubated 1 h with appropriate horseradish peroxidase-conjugated goat antibody. Then, peroxidase activity was developed using the ECL Western Blotting System (Amersham, Rahn AG, Zurich, Switzerland), according to the manufacturer's instructions, and blots were scanned (Arcus II; Agfa, Mortsel, Belgium).

Adhesion assay and apoptosis

This adhesion assay was developed to activate cadherin homophilic ligation on single cells using a specific functional recombinant cadherin protein immobilized to an inert substrate (Fig. 1). Recombinant human cadherin-Fc chimeric proteins containing specific ectodomains for E-, N-, and P-cadherins (hereafter referred to as E-cad/Fc, N-cad/Fc, and P-cad/Fc, respectively) were purchased from R&D Systems (Abingdon, United Kingdom). Glass coverslips or wells of multiwell-printed microscope slides (Thermo Scientific, Braunschweig, Germany) were coated or not with 5 or 25 μg/ml cadherin-Fc chimera, diluted in H_2O, and incubated 18–20 h at 4 C. Then they were rinsed with H_2O and air dried. Islet cells were seeded on these prepared substrates at 30,000 cells/50 μl in appropriate medium supplemented or not with cytokine cocktail (10 ng/ml IL-1β + 5 ng/ml TNFα + 1000 U/ml IFNγ; Invitrogen AG, Basel, Switzerland) as indicated, and incubated at 37 C for 1 or 24 h. Preparations were rinsed to remove unattached cells, and adherent cells were observed and photographed under a Leica DM IL inverted microscope equipped with a digital camera (Leica Microsystems, Renens, Switzerland). For apoptosis analysis, cells were fixed 20 min with 10% methanol-free formalin, rinsed in PBS, and stored at 4 C before to be processed for terminal deoxynucleotide transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick-end labeling (TUNEL) and immunofluorescence. To this end, cells were permeabilized using a solution of 0.1% Triton X-100 in 0.1% sodium citrate (4 min at room temperature), rinsed in PBS and submitted to TUNEL using the *In Situ* Cell Death Detection kit (Roche, Basel, Switzerland) following manufacturer instructions. Cells were rinsed again in PBS, preincubated 20 min at room temperature in PBS containing 0.1% BSA, and exposed 1 h to a guinea pig antiinsulin antibody (Dako) diluted 1:600 in PBS containing 0.1% BSA. Cells were rinsed and exposed 1 h to an Alexa Fluor 488-labeled antiguinea pig antibody (Invitrogen AG). After a last rinsing in PBS, cells were analyzed and photographed using an Axioskop microscope (Zeiss) equipped with a color CCD camera, UV illumination, and filters for blue, red, and green fluorescences. Single and aggregated cells, labeled or not for insulin and positive or negative after TUNEL, were scored.

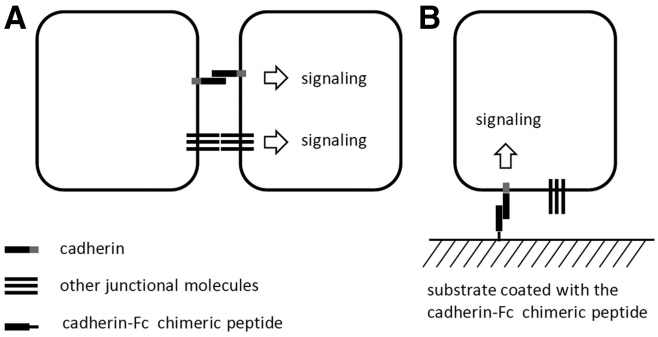

FIG. 1. Diagram of the approach used to study cell adhesion through cadherins. A, In aggregated cells, outside-in signaling is generated by engagement of cadherins and other junctional molecules present at the cell-to-cell contact. B, In single cells adherent to a substrate coated with cadherin-Fc chimeric proteins, outside-in signaling is possible only through the cadherins.

Statistics

Data were expressed as mean ± SEM of n different experiments or islets. Differences between means were assessed either by the Student's *t* test and when required by one-way ANOVA. When ANOVA was applied, Tukey *post hoc* analysis was used to identify significant differences.

Results

Expression of cadherins at the surface of β-cells

Cadherin expressions in islets were analyzed first by immunofluorescence on cryosections of human pancreas. Three cadherin isoforms, E-, N-, and P-cadherins, were stained using FITC-conjugated secondary antibodies, and two islet hormones, insulin and glucagon, were stained using coumarin- and rhodamine-conjugated secondary antibodies, respectively. With regard to E-cadherin, staining was mainly observed at the cell-to-cell contact in both endocrine and exocrine tissue, including acini and ducts. In islets, E-cadherin was clearly expressed at the surface of β-cells and non-β-cells, including α-cells (Fig. 2A). We also observed a cytoplasmic staining in the same cells labeled at the membrane, which could account for synthesis or endocytosis of cadherin. Computer-aided morphometric analysis showed that the pixel intensity of E-cadherin staining, comprising both cytoplasmic and membrane staining, was slightly lower in β-cell compared with α-cell areas (Fig. 2E). E-cadherin staining in α-cells and other endocrine islets cells persisted in type 1 diabetes (T1D) islets where β-cells were virtually absent (Fig. 2C). With regard to N-cadherin, staining was faint in exocrine tissue and mainly restricted to ductal cells. In islets, N-cadherin staining was also observed (Fig. 2B). It predominated at the cell surface but was also observed in cytoplasm. Interestingly, this staining was mainly observed in β-cells and, when present, was faint in α-cell area. Quantitative analysis confirmed that the pixel intensity of N-cadherin was higher in β-cell than α-cell areas (Fig. 2F). As a consequence of this particular distribution of N-cadherin, T1D islets wherein β-cells were absent displayed no N-cadherin staining (Fig. 2D). With regard to P-cadherin, no staining was observed either in islets or in exocrine tissue (data not shown). As positive control for P-cadherin staining, human skin cryosections were processed like were pancreas cryosections. A clear staining was observed at the cell surface of the basal layer of epidermis as previously described (26). Cadherin expressions were also studied in dissociated islet cells. Immediately after enzymatic treatment (1 h), islet cells displayed no staining for N-cadherin, and staining for E-cadherin was restricted to cytoplasmic compartment and small surface areas at the contact between aggregating cells (Fig. 3A). After 24 h of incubation, E-cadherin staining became apparent at the surface of all aggregated islet cells in both β- and non-β-cells. Single cells also displayed a faint surface staining (Fig. 3B). With regard to N-cadherin, staining was less frequent and weaker. When present, it was restricted to β-cells, and virtually never seen on non-β-cells (Fig. 3C). P-cadherin staining was absent in islet cells but was present at the surface of HaCaT cells used as positive control. By Western blotting, E- and N-cadherins were shown in protein extracts of islet cells (Fig. 3D). Using FACS-sorted islet cells as source of protein extracts, we confirmed that N-cadherin was preferentially expressed in β-cells compared with non-β-cells, whereas E-cadherin was similarly expressed in both β- and non-β-cells (Fig. 3D). By double immunofluorescence, we showed that E- and N-cadherins colocalized with β-catenin at the cell membrane of islet cells (Fig. 4), suggesting that cadherins may play a role in signaling mediated by β-catenin in islets.

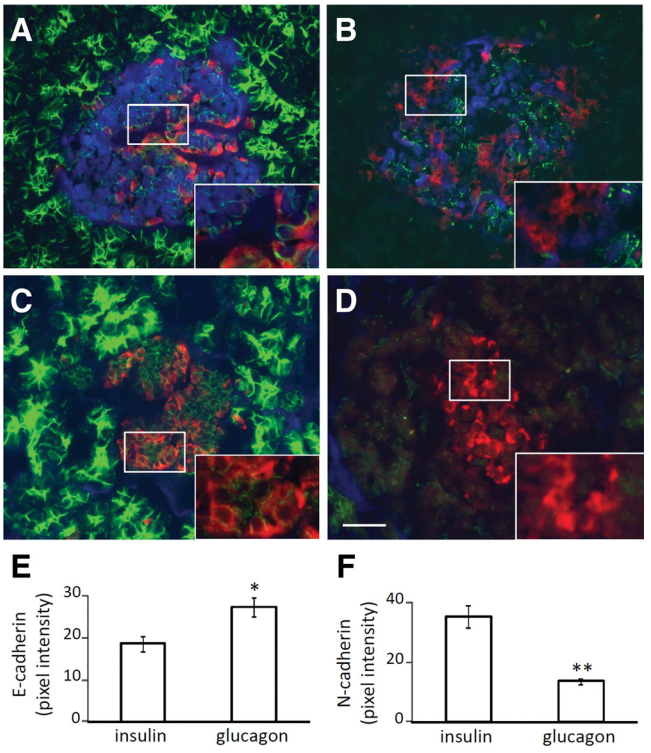

FIG. 2. Expression of E- and N-cadherins in islets. Cryosections from nondiabetic (A and B) and T1D human pancreata (C and D) were stained by immunofluorescence for insulin (*blue*), glucagon (*red*), and either E-cadherin (A and C, *green*) or N-cadherin (B and D, *green*). A, E-cadherin was at cell surface of both β-cells and α-cells. B, N-cadherin was preferentially at the surface of β-cells. C, E-cadherin was at the cell surface of α-cells and other non-β- non-α-islet cells. D, N-cadherin was absent in α-cells. *Scale bar*, 80 μm. *White boxes* indicate fields shown at higher magnification in *lower right corners*. E and F, Pixel intensities of E-cadherin (E) and N-cadherin (F) staining in insulin and glucagon-stained area; *columns* are means ± SEM of 11 islets from two different pancreata; *, $P < 0.005$; **, $P < 0.00005$.

Adhesion of islet cells to cadherin peptides

Three chimeric constructs, E-cad/Fc, N-cad/Fc, and P-cad/Fc, were used to coat coverslips or slides and adhesion of islet cells were tested. Compared with control conditions (Fig. 5A), E-cad/Fc (Fig. 5B) and N-cad/Fc (Fig. 5C) both increased islet cell attachment, because P-cad/Fc (Fig. 5D) had no effect. This effect was on both β- and α-cells. This positive effect of E-cad/Fc and N-cad/Fc on cell at-

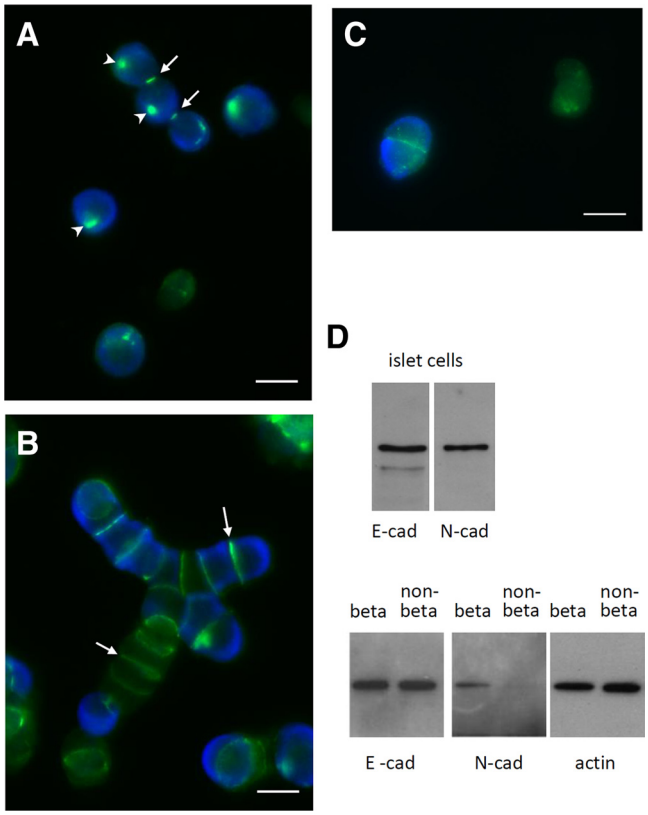

FIG. 3. Expression of E- and N-cadherins in islet cells. A–C, Islet cells were incubated 1 (A) or 24 h (B and C) at 37 C after their isolation by enzymatic treatment and stained by immunofluorescence for insulin (*blue*) and either E-cadherin (A and B, *green*) or N-cadherin (C, *green*). A, E-cadherin was restricted to small cytoplasmic (*arrowheads*) and intercellular contacting areas (*arrows*). B, E-cadherin was intercellular in both β- and non-β-cells (*arrows*). C, N-cadherin was at the surface of β-cells and absent in non-β-cells. *Scale bar*, 15 μm. D, Western blotting showed that E-cadherin (E-cad) and N-cadherin (N-cad) were expressed in islet cells; Western blotting of FACS-sorted islet cells showed that E-cadherin was expressed at similar extent in β- and non-β-cell fractions and that N-cadherin was preferentially expressed in β-cell fraction; actin is shown as control; results are representative of three different experiments for total islet cells and two different experiment for FACS-sorted islet cells.

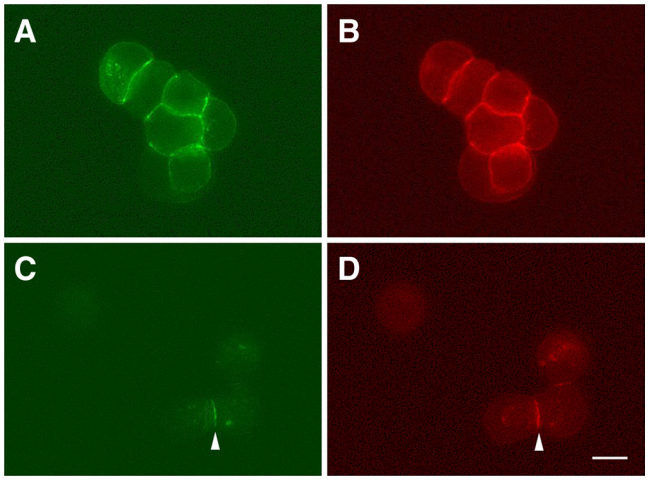

FIG. 4. Colocalization of cadherins and β-catenin in islet cells. Islet cells were incubated 24 h at 37 C after their isolation by enzymatic treatment and double stained by immunofluorescence for cadherins (*green*) and β-catenin (*red*). A and B, Fluorescence views showing aggregated islet cells costained at cell membrane with anti-E-cadherin (A) and anti-β-catenin (B) antibodies. C and D, Fluorescence views showing a cell-cell contacting area (*arrowhead*) costained with anti-N-cadherin (C) and anti-β-catenin (D) antibodies. *Scale bar*, 15 μm.

tachment occurred in the first hour after islet cells were seeded on these substrates (Figs. 5, A–D). We quantified this effect by evaluating the number of cells attached after rinsing per a given microscopic area. Results (means ± SEM of four experiments) were 155 ± 17, 523 ± 59, 255 ± 21, and 159 ± 28 cells, for control, E-cad/Fc ($P < 0.005$, *vs.* control), N-cad/Fc ($P < 0.02$, *vs.* control), and P-cad/Fc, respectively. After a 24-h incubation, differences of cell attachment between chimeric constructs and control were still observed. In addition, after 24 h, E-cad/Fc and N-cad/Fc induced islet cells to flatten and to change their shape, a phenomenon called hereafter cell spreading (Fig. 5, E and F). This effect was clearly apparent with 25 μg/ml E-cad/Fc and observable to a lesser extent with 25 μg/ml N-cad/Fc and 5 μg/ml E-cad/Fc. The percentages of spreading cells were 0, 45, and 17% for control (n = 249 cells from two experiments), 25 μg/ml E-cad/Fc (n = 1127 cells from two experiments), and 25 μg/ml N-cad/Fc (n = 1131 cells from 2 experiments), respectively. Cell spreading was not observed with lower concentration of N-cad/Fc and with P-cadh/Fc (at both high and low concentration).

Cadherin engagement decreases apoptosis of β-cells

Apoptosis of β-cells was analyzed by TUNEL followed by immunofluorescence for insulin. When islet cells were attached to control glass and incubated for 24 h, 12.1 ± 1.8% of single cells stained for insulin after immunofluorescence (β-cells) were TUNEL positive (Fig. 6A). This percentage was significantly decreased (6.1 ± 1.3%, $P < 0.02$) when analysis was limited to aggregated β-cells (Fig. 6B). When cells were attached to E-cad/Fc, the percentage of single β-cells that were TUNEL positive decreased compared with single cells attached to control. This effect was observed at 5 μg/ml and further increased at 25 μg/ml E-cad/Fc (Fig. 6A). With N-cad/Fc, the effect on β-cell apoptosis was observed at 25 μg/ml ($P < 0.05$) and not at 5 μg/ml. By contrast, P-cad/Fc had no effect at either 5 or 25 μg/ml. In aggregated β-cells, where the rate of apoptosis was very low *per se*, neither E-cad/Fc nor N-cad/Fc had an effect (Fig. 6B). We compared the effect of a proapoptotic cytokine cocktail (IL-1β + TNFα + IFNγ) on viability of single *vs.* aggregated β-cells. As expected, this cytokine cocktail increased apoptosis of β-cells, and interestingly, a significant increased ($P < 0.05$) of TUNEL-

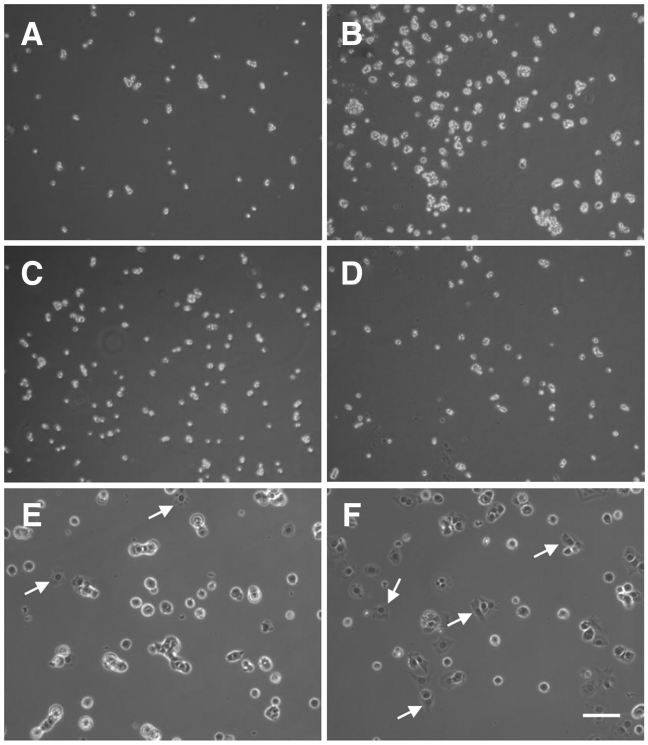

FIG. 5. Adhesion of islet cells to cadherin peptides. Islet cells were isolated, incubated in suspension for 24 h, attached 1 h to control glass (A) or glass coated with 5 μg/ml E-cad/Fc (B), N-cad/Fc (C), or P-cad/Fc (D), and then rinsed and photographed. Alternatively, islet cells were attached 24 h to glass coated with 25 μg/ml N-cad/Fc (E) or E-cad/Fc (F). Compared with control (A), E-cad/Fc (B) and N-cad/Fc (C) increased cell attachment, whereas P-cad/Fc had no effect (D). Images are representative of six to eight different experiments. Cell spreading (*arrows*) was observed when islet cells were attached to 25 μg/ml N-cad/Fc (E) or E-cad/Fc (F). Images are representative of five different experiments. *Scale bar*, 150 μm (A–D) and 60 μm (E and F).

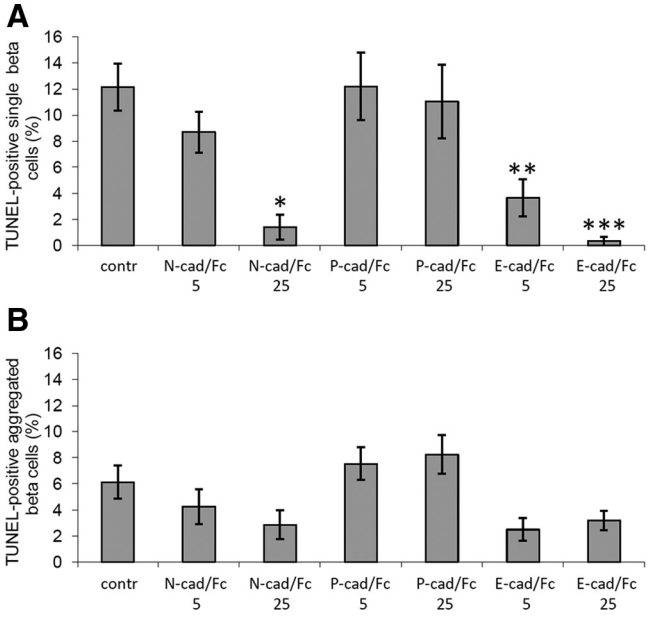

FIG. 6. Effect of cadherin engagement on apoptosis of β-cells. Islet cells were attached to uncoated glass (contr) or glass coated with 5 or 25 μg/ml cadherin peptides. After 24 h, islet cells were submitted to immunofluorescence for insulin and TUNEL. The percentage of TUNEL-positive cells was evaluated among single β-cell (A) and aggregated β-cell populations (B). Data are means ± SEM of seven experiments for controls and cadherin peptides at 5 μg/ml and three experiments for cadherin peptides at 25 μg/ml. Total numbers of single cells analyzed were 959, 841, 304, 775, 341, 843, and 297 for control, N-cadherin 5 μg/ml, N-cadherin 25 μg/ml, P-cadherin 5 μg/ml, P-cadherin 25 μg/ml, E-cadherin 5 μg/ml, and E-cadherin 25 μg/ml, respectively. Total numbers of aggregated cells analyzed were 1559, 1672, 851, 1356, 381, 1751, and 289 for control, N-cadherin 5 μg/ml, N-cadherin 25 μg/ml, P-cadherin 5 μg/ml, P-cadherin 25 μg/ml, E-cadherin 5 μg/ml, and E-cadherin 25 μg/ml, respectively. *, $P < 0.04$; **, $P < 0.03$; ***, $P < 0.02$ *vs.* control. contr, Control.

positive cells was observed in single and not in aggregated β-cells (Fig. 7). The effect of E-cad/Fc on β-cell apoptosis was further evaluated in presence of cytokines. Interestingly, the effect of cytokines on apoptosis of single β-cells attached to control substrate was abolished when cells were attached to E-cad/Fc. By contrast, apoptosis of aggregated β-cells was not affected by E-cad/Fc. These data suggest that cadherin-mediated cell-to-cell contacts protect β-cells from apoptosis, under both control conditions and after exposition to cytokines.

Apoptosis is preferentially observed in E-cadherin-negative cells

When islet cells were incubated 24 h in control conditions and then submitted to immunofluorescence staining for insulin and E-cadherin and labeled by TUNEL, we observed that most islet cells and β-cells displayed a cell-to-cell contact E-cadherin staining and were not labeled by TUNEL. By contrast, TUNEL labeling was observed in aggregated cells (stained or not for insulin) that did not display E-cadherin staining (Fig. 8).

Discussion

It is the first time that expression of cadherins is reported in human islets. E-, N-cadherins, and other adhesion molecules were shown to be expressed in islets of rodent species. Because increasing evidences showed that human islets differ in several respects from rodents, it was legitimate to study the expression of cadherins in human islets. We found that E-cadherin was present in virtually all epithelial cells of human pancreas, including acinar, ductal, and islet cells. Labeling was rather homogeneous among islet cells, even though staining intensity by immunofluorescence was slightly higher in islet areas rich in α-cells, suggesting that E-cadherin is particularly involved in heterotypic contacts between α- and β-cells. With regard to N-cadherin, labeling was faint or absent in exocrine and most islet cells. Higher level of N-cadherin staining was restricted to β-cells that however displayed rather heterogeneous N-cadherin staining intensities. N-cadherin is the most widespread cadherin of the nervous system and is particularly associated with excitatory synapses (27). Interestingly, ro-

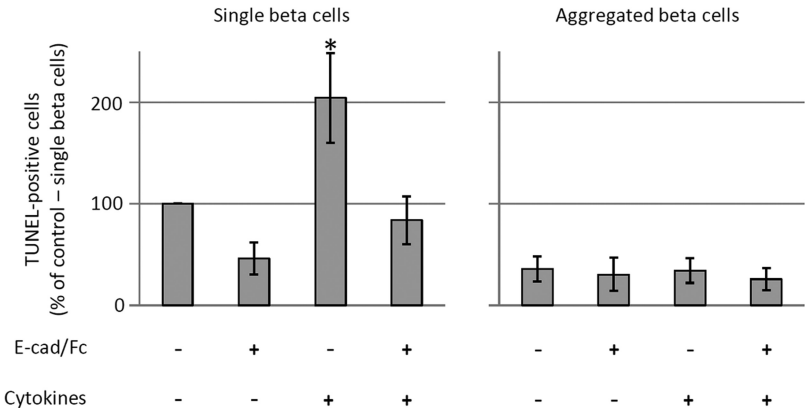

FIG. 7. Effect of E-cadherin engagement on apoptosis induced by cytokines. Islet cells were attached either to control glass or to glass coated with 5 μg/ml E-cad/Fc and incubated 24 h in absence or presence of cytokines (10 ng/ml IL-1β + 5 ng/ml TNFα + 1000 U/ml IFNγ). Islet cells were then stained by immunofluorescence for insulin and TUNEL. The percentage of TUNEL-positive cells was evaluated among single β-cell and aggregated β-cell populations. Due to the great variability between experiments, data are expressed as percentage of single β-cells in control conditions. Data are means ± SEM of five experiments. Total number of cells analyzed per condition was at least 500 and 1000 for single and aggregated cells, respectively. *, $P < 0.03$ compared with the other conditions.

dent islet-cells were shown to express another adhesion molecule highly expressed in nervous system, the neural cell adhesion molecule (NCAM) (28). In addition, a sialylated form of NCAM (polysialylated NCAM) was shown to be expressed in β-cells (29) and not in other islet cells. Altogether, these results are in agreement with the notion of a structural and molecular similitude between β-cells and neurons, which share also elements of the secretory pathway. It would be particularly interesting to understand whether the heterogeneity of N-cadherin expression in human β-cells is physiologically relevant. In rodents, heterogeneous expression of E-cadherin and PSA-NCAM expressions at the surface of β-cells correlated with differences in glucose responsiveness (20, 29). Adhesion molecules play also an important role in islet cell aggregation during rodent pancreas development and in maintaining the unique islet architecture with segregated areas of endocrine cell types (17, 28, 30). Knockdown gene or transgene expression strategies were used to obtain these results, and obviously realization of similar experiments in humans is impossible. The characterization of expression of cadherins in developing islets of fetal pancreas specimens could nevertheless give an insight in the role of cadherins in the development of islet in humans.

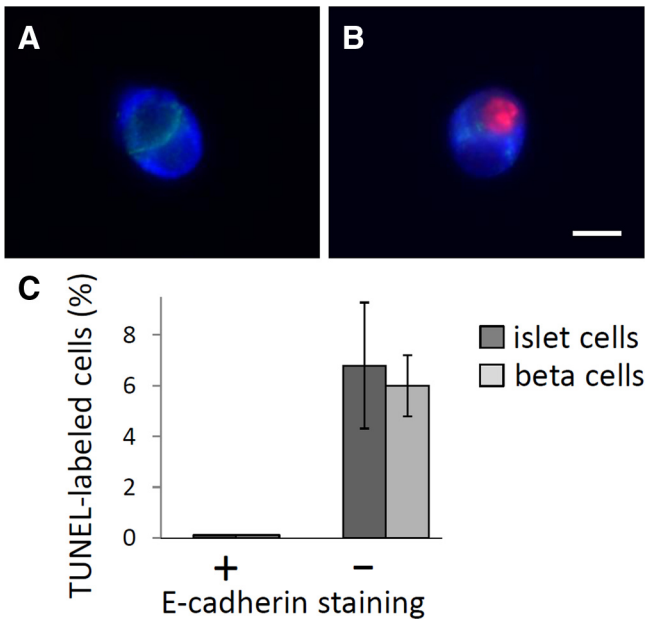

FIG. 8. E-cadherin staining is absent in apoptotic islet cells. A and B, Islet cells cultured 24 h in suspension were stained by immunofluorescence for insulin (*blue*) and E-cadherin (*green*) and TUNEL (*red*). Most aggregated β-cells displayed E-cadherin staining at the cell-to-cell contact area and were not labeled by TUNEL (A); when one β-cell was labeled by TUNEL, E-cadherin staining was absent from the cell-to-cell contact area (B). *Scale bar*, 10 μm. C, The percentage of TUNEL-labeled cells was analyzed in aggregated islet cells or β-cells that were stained (+) or not (−) by immunofluorescence for E-cadherin. Data are mean ± SEM of four experiments.

The cad/Fc peptide approach was specially motivated by the bulk of studies showing the importance of gap junctions in the normal function of β-cells (10, 31). Islet cells of many species, including humans (32), are coupled by gap junction channels made of the connexin36 protein. These channels mediate the coupling of adjacent islet cells and contribute to control islet function, particularly secretory activity of β-cells (31). More recently, gap junctions have been suggested to protect β-cells against streptozotocin and cytokine effects both *in vitro* and *in vivo* (10). In this study, we showed that apoptosis in aggregated cells was lower than in single cells. This cell-to-cell contact effect results certainly from a combination of mechanisms involving gap junction, cadherins, and eventually other molecules. By analyzing single cells adherent to specific cadherin peptides, we can exclude that functional gap junction are formed and that other cell surface molecules are engaged; therefore, the effect observed on apoptosis can be accounted to the solely engagement of specific cadherins.

Under the studied conditions, E-cad/Fc had more effect that N-cad/Fc in protecting β-cells from apoptosis. Furthermore, an association between apoptosis protection and cadherin expression was observed. Indeed, the higher effect of E-cad/Fc correlated with the higher rate of cell adhesion on this peptide compared with N-cad/Fc and also

with higher level of staining for E-cadherin compared with N-cadherin at the surface of β-cells. Obviously, differential efficiencies of cad/Fc peptides must be also considered.

Our results showing that cadherin-mediated cell-to-cell adhesion is important in maintaining viability of islet cells may have some relevance in islet transplantation. First, cell-to-cell adhesion is disrupted by the isolation procedure and other manipulations required in islet transplantation. For instance, islet cells are submitted to enzymatic treatment that may affect or even destroy cell surface molecules, including cadherins. In addition, disruption of the islet cell surface arrangement may occur after islet transplantation, as a result of the inflammatory phenomena elicited at the site of engraftment and leading to primary nonfunction or of allogeneic immune reactions leading to graft rejection. Because these events are known to cause a massive loss of β-cell mass by apoptosis, we speculate that cadherins can be involved in the loss of islet cell viability occurring in these processes. In both allogeneic rejection and T1D, cytotoxic T lymphocytes are able to activate proapoptotic cascades via their secreted enzymes, such as the subfamily of serine proteinases granzymes. Granzymes have been identified also in extracellular locations, where they have been shown to cleave molecules involved in cell adhesion and therefore causing cell death by anoikis (33). That cadherins should be destroyed by these proteases has not yet been shown. Nevertheless, our results demonstrate that cadherins at the surface of islet cells can be easily destroyed by the prototype serine protease trypsin, an enzyme with the same active site as granzymes.

Interaction between the integrin αEβ7 (CD103) and E-cadherin can be accounted for another mechanism involved in T-cell cytotoxicity. The conventional viewpoint is that interaction of CD103 with E-cadherin expressed on grafted epithelial cells induces the cytolytic activity of T cells. Using a mouse model of pancreatic islet transplantation, Feng *et al.* (34) demonstrated that wild-type mice rejected islets transplanted under the renal capsule and that this effect correlated with the appearance of T cells expressing CD103 at the graft site. In accordance, the majority of islet allografts transplanted into CD103 knockout hosts mice survived indefinitely (34). These data emphasize the hypothesis that CD103-E-cadherin interaction is required for T cell-mediated destruction of graft epithelial elements.

Cadherin disengagement has been linked to tumor progression and activation of cell proliferation (35, 36). In islet cells, a role of E-cadherin in β-cell proliferation has been suggested (37). The cadherin-Fc peptide approach has been used by others to study effect of E-cadherin on cell proliferation (38) and can be surely transposed to further study direct cadherin effect on β-cell proliferation. Unfortunately, human β-cells do not proliferate *in vitro* (25); consequently, such a study should be envisaged in rodent β-cells.

The downstream signaling pathways involved in cadherin-mediated survival of islet cells have not been investigated in the present study, and only few works explored this issue in other cell types. For instance cadherin engagement has been shown to promote tumor cell survival via activation of Rho-family GTPases that furthermore transduce the signal to transcription factors, such as the signal transducer and activator of transcription-3 (39). Signal transducer and activator of transcription-3 activity is routinely observed in cancers by promoting cell proliferation and preventing apoptosis (40).

In conclusion, these data show that E- and N-cadherins are expressed at the surface of human β-cells and that these adhesion molecules are involved in the maintenance of β-cell viability. Thus, preservation of cadherin expression in transplanted islets might represent a novel strategy to prevent primary graft nonfunction and minimize cell damage induced by allogeneic graft rejection.

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