Original Article

Integrin Signaling via RGD Peptides and Anti-β1 Antibodies Confers Resistance to Apoptosis in Islets of Langerhans

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Islet transplantation is associated with a high rate of early graft failure caused by early immune attack and poor functionality of islets. Apoptosis of islet cells appears soon after islet isolation and primarily involves the β-cell. The purpose of this study was to determine the effect of ligation to extracellular matrix (ECM) proteins on survival of the islets of Langerhans following islet isolation. Islets that had been cultured for 24 h on collagen type I showed an islet survival of 59.7 ± 8.7%, while islets that had been cultured on collagen type IV and laminin showed an islet survival of 88.6 ± 10.3 and 94.3 ± 5.6%, respectively. Islets that had been pretreated with anti-β1 antibodies and argenin-glycin-aspartic acid (RGD) peptides showed a decrease in the level of apoptosis by a factor of 2.5 and 3.1, respectively, and an increase of phospho-Akt Ser473 activity by a factor of 1.6 and 2.2, respectively, and an increase of phospho-Akt Ser473 activity by a factor of 2.5 and 3.1, respectively, compared with untreated islets. When detached from their natural ECM surrounding in the pancreas, islet cells undergo apoptosis, unless islets are cultured on collagen IV or laminin or treated with anti-β1 integrin antibodies or RGD peptides to mimic ECM ligation. These results indicate that inhibition of anoikis may offer opportunities to improve function and viability of islet cells. Diabetes 55:312–317, 2006

Transplantation of islets of Langerhans has the potential to become a widely applicable treatment for type 1 diabetes. Unfortunately, islet transplantation is associated with a high rate of early graft failure (1–4), which is caused by early immune attack, poorly functioning islets that were presumably damaged during isolation, or both. Apoptosis of islet cells appears soon after islet isolation and primarily involves the β-cell (5).

During islet isolation by collagenase treatment, during purification, and during pretransplanting culture, the microenvironment of the islet is destroyed. Extracellular matrix (ECM) is an important component of the islet microenvironment that prevents cellular stress, which could impair β-cell function and survival (6,7). The islet basement membrane is composed of the ECM proteins laminin, fibronectin, and collagen types I, III, IV, and V (8–9). Cell-matrix interactions are necessary because they provide signals for proliferation, differentiation, and migration (10). These interactions are mediated by integrins, which are a diverse class of αβ heterodimeric transmembrane receptors that form ligands for the ECM (10–12).

Cells disconnected from their ECM can undergo apoptosis by an integrin-mediated death signal called anoikis (6,13,14). The pancreatic cells express the integrins αβ1, αβ5, and αβ3 (7,15), which are also known to bind argenin-glycin-aspartic acid (RGD) sequences on ECM molecules. Cell attachment mediated by the αβ1 and the αβ3 integrin promotes cell survival by upregulation of Bcl-2 (16,17). Integrins are believed to mediate survival by activating a specific signaling pathway. One of its ligands is integrin-linked kinase (ILK), which can phosphorylate β1 and β3 integrins (18). ILK is believed to function as the effector of phosphatidylinositol 3-kinase. Protein kinase B/Akt on serine 473 will be directly phosphorylated (19), and it suppresses apoptosis (20–22).

In the present study, we investigated how survival of islets of Langerhans is influenced by either anti-β1 integrin antibodies or RGD peptides. Our results show that apoptosis of β-cells in islets of Langerhans is suppressed by both anti-β1 antibodies and RGD peptides.

RESEARCH DESIGN AND METHODS

Islet isolation and purification with magnetic retraction. Inbred male albino Oxford (AO) rats (n = 15) weighing 200–250 g were obtained from Harlan (Horst, the Netherlands). All animals received humane care in compliance with the National Research Council’s criteria for humane care as outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (1985). They were anesthetized by a subcutaneous injection that contained a mixture of hypnorm and dormicum in PBS. The islets of Langerhans were isolated with the magnetic retraction technique (23). In brief, the descending aorta was perfused with a freshly sonicated 1.25% suspension of Fe3O4 (Sigma-Aldrich, Zwijndrecht, the Netherlands) in PBS, after the thoracic aorta had been clamped. To provide outflow, an incision was made in the distal vena cava. The pancreas was rapidly excised and cut into small pieces (2 mm3) with single-edged razor blades. Islets were isolated from the surrounding exocrine tissue by enzymatic digestion with 0.7 mg/ml collagenase P (Boehringer Mannheim, Almere, the Netherlands) in 25 mmol/l Krebs-Ringer-Hepes solution containing 10% BSA at 37°C for 15 min. The digest was passed through a metal screen with 210-μm diameter pores. The filtrate that was thus obtained was passed through a 50-μm nylon screen (Becton Dickinson, Alphen aan den Rijn, the Netherlands), and the residue was collected. The falcon tube was immediately placed next to the magnetic
pole of the Dynal Magnetic Particle Concentrator (Dynal AS, Oslo, Norway) for 30 s. Successful magnetic purification was confirmed by visible accumulation of black specks on the inner sidewall of the tube next to the magnet. **Histological analysis of islets and pancreata.** Pancreata and isolated islets from the AO rats were fixxed in 4% paraformaldehyde overnight and dehydrated in a graded ethanol series. Consecutive sections were immunostained for the ECM markers laminin (dilution 1:100; Dako, Glostrup, Denmark) and collagen type IV (dilution 1:100; Sanbio, Uden, the Netherlands). Antigen retrieval was performed by boiling the deparaffinized sections for 10 min in 0.01 mol/l citrate buffer at pH 6.0. All immunohistochemicals were diluted in PBS containing 1% (wt/vol) BSA. The sections were incubated with the primary antibody overnight. The sections were washed three times with PBS for 5 min. Bound antibodies were visualized with peroxidase-labeled swine anti-rabbit IgG (Dako) and developed with H2O2 and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as chromogen. The sections were counterstained with hematoxylin.

**Adhesion assay.** To delineate the islet-ECM interaction, an adhesion assay was used as previously described (7). We have adapted this technique to determine whether islets bind specifically to dishes coated with different types of ECM. Islets were cultured on 24-well culture plates that had been noncoated or precoated with collagen type I, collagen type IV, laminin, or fibronectin (Biocoat, Micronic systems, Lelystad, the Netherlands). Approximately 20 islets per well were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% oxygen. After incubation for 24 h, the number of attached islets was established with a Leitz inverted phase contrast microscope.

**Culture of islets.** A total of 150 islets were incubated with 10 μg hamster anti-β1 (IgM) antibodies (Pharmingen, Palo Alto, CA) for 10 min at 37°C or 0.1 mmol/l RGD peptides for 30 min at 37°C or they remained untreated. Islets were suspended in RPMI-1640 medium (Sigma-Aldrich) containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 mg/ml insulin, and 10% FCS, and they were plated at a density of 100 islets/well. These 24-well culture plates were precoated with (12 mg/ml) polyhydroxyethylmethacrylate (polyHEMA) (Sigma-Aldrich) to prevent attachment of the cells (24). The islets were cultured for 24 h at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

**Islet viability assay.** After 1 day, cultured islets were incubated with SYTO and with DEAD Red for 1 h (Molecular Probes, Leiden, the Netherlands) and examined by confocal microscopy (Zeiss, Sliedrecht, the Netherlands). Viable cells stained green, and nonviable cells were seen as red-stained nuclei.

**Lymphocyte isolation and culture.** Lymphocytes, which do not require attachment to ECM for survival, were treated with RGD peptides and served as positive control for caspase-3 activation. Rat thymus glands (n = 3) were removed from anesthetized animals, minced, and washed in RPMI-1640 medium to obtain lymphocytes. The cell suspension was filtered through a 150-μm-pore diameter nylon sieve (Becton Dickinson) and centrifuged at 200g for 10 min. The precipitated cells were resuspended in fresh RPMI-1640 medium. A lymphocyte suspension of 1 × 10⁶ viable cells per ml was incubated with 0.1 mmol/l RGD peptides for 30 min. Lymphocytes were suspended in RPMI-1640 medium and cultured on 24-well polyHEMA-coated plates for 18 h. The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

**Detection of apoptosis by active caspase-3 and survival by phospho-Akt Ser 473 immunostaining.** Islets were cultured with or without anti-β1 integrin MoAbs or with RGD peptides for 18 h, fixed in 4% paraformaldehyde overnight, and dehydrated in a graded ethanol series. The islets were embedded in paraffin and cut into 3-μm sections. Active caspase-3 was detected with diluted (1:3,000) rabbit polyclonal antibody CM1 (Becton Dickinson) (25). The diluted (1:50) rabbit anti-phospho-Akt Ser 473 antibody (Westburg, Leusden, the Netherlands) was used as a survival marker. The antigen was retrieved by boiling the deparaffinized sections for 10 min in 0.01 mol/l citrate buffer at pH 6.0. Overnight incubation with the primary antibody was followed, after washing, by 1 h of incubation with diluted (1:100) biotinylated swine anti-rabbit IgG (Dako). Finally, the sections were treated with peroxidase-labeled streptavidin-biotin-peroxidase complex (Dako) and developed with H2O2 and 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. For negative controls, the primary antibodies were omitted. The percentage of caspase-3- or phospho-Akt Ser 473-positive cells was determined by calculating the number of caspase-3- or phospho-Akt Ser 473-positive cells per islet (three fields per section were measured at a magnification of 400×).

**Statistical analysis.** Differences in islet adhesion and islet survival after different islet treatments were compared with the Student’s t test. P < 0.05 was considered to be statistically significant. All data are expressed as means ± SD.

**RESULTS**

**Expression of ECM proteins.** Immunohistochemical localization of laminin (Fig. 1) and collagen type IV (not shown) demonstrated that they were components of the

**FIG. 1.** Islets of Langerhans stained for laminin (brown). A: In situ. B: After 1 day of culture. C: After 7 days of culture. D: After 1 month of transplantation. The black spots indicate the presence of iron oxide (magnification 400×).

**FIG. 2.** Survival of the islets of Langerhans is dependent on attachment of islets of Langerhans to the pancreatic ECM. Islets were cultured on collagen type I, collagen type IV, laminin-1, or fibronectin. The number of surviving islets was determined after 24 h of culture. Data are expressed as means ± SD. *P < 0.03 compared with islets cultured on collagen I.
peribasement membrane of the islet and that these ECM components were observed within the islet only along capillaries (Fig. 1A). The basement membrane of isolated islets was lost immediately after enzymatic digestion (Fig. 1B). After the islets had been cultured for 7 days, laminin and collagen IV were no longer found within the islet (Fig. 1C). After islet transplantation, the detection of laminin and collagen IV returned to its original distribution (Fig. 1D).

**ECM adhesion–induced survival.** We examined the percentage of islet survival after a 24-h culture period to determine the influence of several components of the ECM on survival of islets in vitro. Islets cultured on collagen type I showed an islet survival after 24 h of 59.7 ± 8.7%, while islets cultured on collagen type IV, laminin, and fibronectin showed an islet survival rate of 88.6 ± 10.3%, 94.3 ± 5.6%, and 85.6 ± 11.2%, respectively (P < 0.03) (Fig. 2). Attachment of islets to fibronectin could be inhibited to a certain extent by the addition of the integrin-binding peptide RGD and the integrin-binding antibody against α5β1 (P < 0.03). The control peptide RGE and the anti-αvβ3 antibody had no influence on islet adhesion (Fig. 3).

**RGD peptide–induced prevention of anoikis.** The effect of RGD peptides on rat islet-cell viability was measured by confocal microscopy analysis after propidium iodide staining. After 24 h of culture, there was a decrease in dead cells in the RGD-treated islets, while the amount of dead cells in the untreated islets remained constant (Fig. 4).

**Anti-β1 antibodies and RGD peptides prevent activation of caspase-3.** We determined the amount of active caspase-3-positive cells per islet by immunostaining to investigate the influence of the presence of the anti-β1 integrin antibodies and RGD peptides on the caspase-3 activity. The percentage of cells per islet that underwent apoptosis after different treatments was microscopically analyzed after caspase-3 immunostaining. In untreated islets that had been cultured for 1 day, a percentage of 30.7 ± 0.3% of cells per islet stained positively for caspase-3, which is an indication of apoptosis. In contrast, treatment with 10 μg anti-β1 antibodies rendered a percentage of 12.4 ± 1.3% positive caspase-3 cells per islet, which represents a decrease in the level of apoptosis by a factor of 2.5 (P < 0.01). After treatment with 0.1 mmol/l RGD peptides, 9.9 ± 2.2% of cells per islet stained positively for caspase-3, which represents a decrease in the level of apoptosis by a factor of 3.1, in contrast to untreated islets (P < 0.01) (Fig. 5A).

As a positive control, induction of apoptosis was found when lymphocytes were treated with RGD peptides for 18 h. As a result, 18.0 ± 4.8% of lymphocytes stained positively for caspase-3, in contrast to 4.3 ± 1.2% in untreated lymphocytes (P < 0.05) (Fig. 5B).

**Integrin activation increases phospho-Akt Ser 473 activity.** Because integrin receptor activation can lead to ILK-mediated phosphorylation of Akt on serine residue 473, we determined if anti-β1 antibodies or RGD peptides could induce Akt phosphorylation in islet cells. We deter-

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**FIG. 3. Adhesion of islets to fibronectin is integrin mediated.** Islets were cultured on fibronectin in the presence of RGD, argenin-glycin-glutamic acid (RGE), anti-α5β1, and anti-αvβ3 antibodies. Adhesion of the islets was determined after 1 h culture. Data are represented as boxplots. *P < 0.03 compared with untreated islets.

**FIG. 4. RGD peptides confer survival to islets of Langerhans.** Islets of Langerhans were either not treated or treated with RGD peptides and were cultured on polyHEMA (to prevent attachment) for 24 h. Viable cells were visualized with SYTO (green) and dead cells were visualized with DEAD Red. Note the increased survival of cells from islets that were treated with RGD peptides.
mined the amount of phospho-Akt Ser 473–positive cells per islet by immunostaining. Only 24 ± 1.9% of the cells of untreated islets stained positive for phospho-Akt Ser 473, whereas 76.8 ± 22.3% of the cells per islet pretreated with anti-β1 antibodies stained positive for phospho-Akt Ser 473, and 69.7 ± 12.1% of the cells per islet pretreated with RGD peptides stained positive for phospho-Akt Ser 473 (P < 0.05) (Fig. 6). We concluded that phosphorylation of Akt Ser 473 had increased through the ligation of integrins by either anti-β1 antibodies or by RGD peptides.

**DISCUSSION**

Islet transplantation is associated with a high rate of early graft failure. A large percentage of islet transplants are lost early, within weeks after grafting from the implant. Several causes have been held responsible for this phenomenon, including islet death–elicited recurrence of autoimmunity, delayed vascular connection resulting in prolonged hypoxia, tissue factor production resulting in activation of coagulation, and inflammation (26–28). Furthermore, the quality of islet preparation might play a fundamental part in determining the outcome of the graft. Islet isolation, however, exposes the islet to various forms of cellular stress, including disruption of the cell–matrix relationship, an event that is associated with apoptosis (6,7,29–31). The destruction of the islet microenvironment subjects the islets to a type of cellular stress that could impair β-cell function and survival. The cell–matrix relationship is characterized by interaction between cell surface integrin receptors and matrix molecules of the surrounding basement membrane.

In this study, we observed the loss of the periinsular...
basement membrane after islet isolation as reported by Wang and Rosenberg (7). Cells disconnected from their ECM are prone to undergo apoptosis by an integrin signal called anoikis (14,32,33). This anoikis pathway led us to test islet survival in vitro after culturing islets on different components of the ECM. We showed that islets cultured on collagen type I are more susceptible to apoptosis than islets cultured on collagen type IV, laminin, or fibronectin. Others showed that cell loss caused by apoptosis is the first step in the process of islet-to-cyst formation. This is a process that takes place when islets are embedded in collagen I gel. It does not take place when they are embedded in laminin (34). This may be the reason why the rate of survival in the islets that were cultured on collagen type I is lower than that of the islets that were cultured on laminin or collagen type IV. We showed that the presence of ECM proteins is important for the survival of islets. According to the reported low rates of apoptosis of cultured islets embedded in the ECM, free cultured islets had an extraordinary rate (>90%) of apoptotic death after 48 h culture (6).

Islet cells express integrins α3, α5, and αv and colocalize closely with the distribution of insulin immunoreactivity (7,15). These particular integrins are known to bind laminin, collagen, and fibronectin (12) and could therefore be involved in ECM-dependent signal transduction in islet cells. The expression of these integrins on islets appeared to be reduced after isolation (7,15). However, in this study we could not detect any distribution of the αv integrin in the islets of Langerhans (data not shown). Wang and Rosenberg (7) earlier described species differences in integrin expression. The αv integrin expression was only found in human and canine islets of Langerhans (13). This explains that anti-αvβ3 antibodies had no influence on rat islet adhesion to fibronectin in our study. It is known that interaction with the integrins α5β1 and αvβ3 supports cell survival through the upregulation of the expression of bcl-2, an important regulatory protein that inhibits apoptosis, by downstream signaling that primarily occurs through a phosphotydininositol-3-kinase/Akt-related pathway (16, 17). We hypothesized that this antiapoptosis signal, which is usually generated by ECM molecules, can be mimicked by RGD peptides binding to the α5β1 integrins. We observed a decrease in caspase-3 activity and an increase of phospho-Akt Ser 473 activity when islets were treated with RGD peptides or antibodies against the β1 integrin chain for 24 h, in contrast to untreated islets. These islets were cultured on polyHEMA to prevent attachment of the islets. These results strongly suggest that integrins, particularly those containing α5 and β1 subunits, act as RGD receptors that lead to phosphorylation of Akt on serine 473 and therefore suppress apoptosis in islets. ILK is a serine/threonine kinase that associates with the β1 integrin and β3 subunit (18,35) and that is able to promote phosphorylation of Akt on Ser 473 (21,22). Whether ILK will be activated after islets have been treated with RGD peptides or anti-β1 antibodies is an interesting question for future studies. The observation that cells in the center of the islet are protected against apoptosis after treatment with ligands for certain integrins lends support to the notion that the integrin ligation by, for example, ECM acts as a rescue signal for those cells in the periphery. Several studies have shown that small peptides containing RGD motifs can activate cytosolic caspase-3 by direct interaction with the RGD recognition site of caspase-3 (36–38). The RGD peptides enter the cell by a passive, nonintegrin-dependent pathway. Apoptosis that is induced by RGD peptides has most frequently been described in lymphocytes and immortalized cell lines. To confirm these data, we also treated lymphocytes with RGD peptides in the current study and indeed found induction of apoptosis. Since lymphocytes do not require integrins for attachment to RGD sequences on the ECM, exogenous RGD peptides enter these cells instead of binding to their outer membranes. These results indicate that activation of integrins by integrin-binding peptides or antibodies can suppress apoptosis in anchorage-dependent cells, like islets of Langerhans.

In summary, isolated islet grafts show a high level of nonviability and function loss due to an apoptotic mechanism described for anoikis. The studies described in this article indicate that apoptosis of free islet grafts can be inhibited by the use of anti-β1 antibodies or RGD peptides. These results indicate that inhibition of anoikis may offer opportunities to improve function and viability of islet cells.

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