

Impact of Defined Matrix Interactions on Insulin Production by Cultured Human β -Cells

Effect on Insulin Content, Secretion, and Gene Transcription

Thomas Kaido,¹ Mayra Yebra,¹ Vincenzo Cirulli,¹ Christopher Rhodes,² Giuseppe Diaferia,¹ and Anthony M. Montgomery¹

The impact of extracellular matrix on insulin production needs to be understood both to optimize the derivation of functional β -cells for transplantation and to understand mechanisms controlling islet neogenesis and glucose homeostasis. In this study, we present evidence that adhesion to some common matrix constituents has a profound impact on the transcription, secretion, and storage of insulin by human β -cells. The integrin-dependent adhesion of fetal β -cells to both collagen IV and vitronectin induces significant glucose-independent insulin secretion and a substantial reciprocal decline in insulin content. Collagen IV, but not vitronectin, induces comparable responses in adult β -cells. Inhibition of extracellular signal-regulated kinase activation abrogates matrix-induced insulin secretion and effectively preserves the insulin content of adherent β -cells. Using real-time PCR, we demonstrate that adhesion of both fetal and adult β -cells to collagen IV and vitronectin also results in the marked suppression of insulin gene transcription. Based on these findings, we contend that integrin-dependent adhesion and signaling in response to certain matrices can have a significant negative impact on insulin production by primary human β -cells. Such responses were not found to be associated with cell death but may precede β -cell dedifferentiation. *Diabetes* 55: 2723–2729, 2006

Integrins are a family of heterodimeric transmembrane adhesion molecules that serve to integrate a cell's interior machinery with the extracellular environment. Such integration is achieved through the binding of extracellular matrix components and the sub-

sequent activation of intracellular signaling elements (1). Integrins have been implicated in a plethora of processes required for normal development, including cell survival, proliferation, cytodifferentiation, migration, and spatial segregation (1,2).

Complex matrices and individual extracellular matrix components have been shown to strongly affect many aspects of β -cell function, including motility (3,4), survival (5,6), proliferation (7,8), and differentiation (9,10). Several recent reports have shown that matrix interactions can also influence insulin secretion (4,11,12). A complex matrix rich in laminin-5 (804G-matrix) has been shown to potentiate insulin secretion in response to glucose (11,12), and we have shown that common constituents of basement membranes, including collagen IV, induce significant insulin secretion by human fetal β -cells (4).

Although matrix interactions have been shown to be beneficial for insulin secretion in the short term (4,11,12), there is less certainty as to the long-term impact of matrix interactions on insulin content and gene expression. Collagen, matrigel, and fibrin gels have all been reported to induce or maintain insulin content or secretion (13–16). However, others have shown that long-term exposure to both purified and complex matrices, particularly in monolayer culture, results in a significant loss of insulin secretion, message, or content (7,17–19). Interpreting the role of matrix in these long-term studies is complicated by the addition of different exogenous growth factors or serum, which may themselves influence insulin transcription.

The purpose of this study was to define the impact of individual matrix interactions on insulin production by cultured human β -cells. To do this, we have exploited assays that allow the rapid and controlled interaction of β -cells with individual matrices in the absence of potentially confounding factors such as serum or exogenous growth factors. We show that adhesion of primary human β -cells to substrates such as vitronectin and collagen IV results in a dramatic loss of insulin content and insulin production. We show that such losses can be attributed to protracted, extracellular signal-regulated kinase (ERK)-dependent insulin secretion and simultaneous suppression of insulin gene transcription. The observed loss of insulin production was not associated with cell death but may precede β -cell dedifferentiation and anchorage-dependent growth. These observations have important implications

From the ¹Islet Research Laboratory at the Whittier Institute for Diabetes, Department of Pediatrics, University of California at San Diego, La Jolla, California; and the ²Pacific Northwest Research Institute and Department of Pharmacology, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Anthony M. Montgomery, Islet Research Laboratory at the Whittier Institute for Diabetes, Department of Pediatrics, University of California at San Diego, 9894 Genesee Ave., La Jolla, CA 92037. E-mail: ammontgo@ucsd.edu.

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ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; JNK, c-Jun NH₂-terminal kinase; mAb, monoclonal antibody; pAb, polyclonal antibody.

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for the empirical design and optimization of protocols for the ex vivo expansion of β -cells for transplantation.

RESEARCH DESIGN AND METHODS

Monoclonal antibodies (mAbs) to $\alpha_1\beta_3$ (LM609), $\alpha_4\beta_5$ (PIF6), and β_1 (P4C10) were from Chemicon (Temecula, CA). VNR (vitronectin receptor), anti- α , polyclonal antibody (pAb), was generated at the Scripps Research Institute (La Jolla, CA). A guinea pig pAb to insulin was obtained from DakoCytomation (Carpinteria, CA). c-Jun NH₂-terminal kinase (JNK) inhibitor SP600125, p38 inhibitor SB203580, ERK-1/2 inhibitors PD8059 and U0126, and the analog control U0124 were all obtained from Calbiochem (San Diego, CA). Vitronectin and fibronectin were from Chemicon, and collagen IV and entactin-free laminin-1 were purchased from BD Biosciences (Bedford, MA). Collagen I was from Upstate Cell Signaling (Lake Placid, NY).

Derivation of fetal and adult β -cells. Fetal pancreata (20–24 weeks) were obtained from ABR (Alameda, CA). Islet-like cell clusters were generated and cultured in suspension for 2 days as previously described (17,20). Islet-like cell clusters were then transferred and cultured for 4 days on dishes coated with HTB-9 matrix (18) in the presence of 10% fetal bovine serum and hepatocyte growth factor (10 ng/ml) essentially as reported (7,17). Islet-like cell cluster culture on HTB-9 matrix results in some loss of insulin expression (7) but also promotes a gradual transition from spheroidal aggregates to monolayers, which can then be harvested as single cells without compromising β -cell viability. Harvesting was performed using a 0.025% trypsin-versene solution (Gibco). Harvested cells typically contained 85–90% pancreatic epithelial cells and 1–2% insulin-positive β -cells. Human adult islets (age 45–56 years) of 30–40% purity were obtained through the Human Islet Distribution Program (City of Hope National Medical Center, Duarte, CA). Islet clusters were expanded on HTB-9 matrix for 3–4 days and were harvested as described above for fetal cells.

Matrix adhesion, ERK inhibition, and insulin release. Fetal or adult cells were added to 96-well high-binding plates (Costar) precoated overnight (4°C) with equimolar amounts of vitronectin, collagen IV, collagen I, fibronectin, or laminin-1 (75 nmol/l). Additional cells were added to 96-well plates coated with HTB-9 matrix essentially as previously described (18). Control wells were precoated with poly-D-lysine (10 μ g/ml) or BSA (5% in PBS). Fetal cells were added in fibroblast basal media (BioWhittaker) and adult cells in glucose-free Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 2.8 mmol/l glucose. Both of these adhesion buffers were further supplemented with 0.5% BSA and 0.4 mmol/l MnCl₂. Some cells were pretreated with U0126 (25 μ mol/l), PD98059 (50 μ mol/l), SP600125 (20 μ mol/l), or SB203580 (10 μ mol/l) for 20 min before adding cells and inhibitors to precoated wells. Control cells were treated with DMSO vehicle or with control compound U0124 (25 μ mol/l). Cells were added at 1.5×10^4 cells per well, and supernatants were collected 90 min after plating. Supernatants were centrifuged to remove cells or debris and were assayed for insulin by enzyme-linked immunosorbent assay (ELISA; Alpco Diagnostics, Windham, NH).

Matrix adhesion, ERK inhibition, and insulin content. Fetal or adult cells were added to 96-well plates precoated with HTB-9 matrix, vitronectin (75 nmol/l), collagen IV (75 nmol/l), or BSA (5%). Fetal and adult cells were added at 1.5×10^4 cells per well in fibroblast basal media and Dulbecco's modified Eagle's medium adhesion buffers, respectively. Some cells were pretreated with U0126 (25 μ mol/l) or DMSO vehicle alone for 20 min before adding cells and reagents to coated wells. After 1.5 and 18 h, cells attached to collagen IV, vitronectin, or HTB-9 matrix were lysed in situ using a cell lysis buffer from Cell Signaling Technologies (Danvers, MA). Nonadherent cells on BSA-blocked plastic were harvested and lysed in the same manner. The insulin content of lysates was determined by ELISA.

Adenovirus infection and insulin release. Fetal islet-like cell clusters were cultured on HTB-9 matrices for 2 days before infection with an adenovirus expressing green fluorescent protein (GFP) and a dominant-negative form of ERK-2 (Ad-DN-ERK) (21). Control cells were infected with GFP alone (Ad-GFP). The origin and generation of these constructs has been described previously (21,22). Cells were infected at a multiplicity of infection of 100 for 16 h before washing and further culture for an additional 24 h. After this time, the number of GFP and insulin double-positive cells was typically 40–45%. Infected cells were added to 96-well plates previously coated with vitronectin (75 nmol/l) at 1×10^4 cells per well in the fibroblast basal media adhesion buffer. Supernatants were collected 90 min postseeding and were assayed for insulin by ELISA.

Contribution of integrins to insulin release. The contribution of integrins to insulin release was assessed using antibodies LM609 (40 μ g/ml), PIF6 (40 μ g/ml), P4C10 (40 μ g/ml), and VNR (1:15 dilution). Fetal cells were harvested and pretreated with these antibodies for 20 min before the addition of both cells and antibodies to 96-well plates previously coated with vitronectin or poly-D-lysine. Cells were added at 1×10^4 cells per well in the fibroblast basal

media adhesion buffer. Culture supernatants were collected 90 min postadhesion and were assayed for insulin by ELISA.

Changes in insulin release and content with time on vitronectin. Fetal cells were added to 96-well plates coated with vitronectin (75 nmol/l). Cells were added at 1×10^4 cells per well in fibroblast basal media adhesion buffer. After 1 h, nonadherent cells were removed and the buffer replaced. The insulin content of these adherent cells was then assessed every hour together with the insulin content of cells detaching from the vitronectin over the same time course. At each time point, the supernatant was removed, and the adherent cells were lysed in 100 μ l of lysis buffer (Cell Signaling Technologies). Detached cells present in the supernatant were pelleted and lysed in the same buffer. Insulin released into the supernatant was assessed over the same time course but in separate plates. Cells were seeded as described above, and supernatant was collected from individual wells and fresh buffer added every hour. The insulin content of lysates and supernatants was determined by ELISA.

Matrix adhesion, ERK inhibition, and number of insulin-positive cells. Fetal cells in fibroblast basal media adhesion buffer were added to 96-well plates precoated with vitronectin (75 nmol/l) or collagen IV (75 nmol/l). Some cells were treated with inhibitors of ERK-1/2, JNK, or p38 or were infected with a dominant-negative ERK construct as described above. Adherent cells were subsequently stained for insulin after 1.5 or 18 h. Staining was performed using a guinea pig pAb to insulin (dilution 1:40; DakoCytomation) as previously described (3). Insulin-positive cells per field were counted using an inverted microscope, a 20 \times objective, and an ocular grid. Treatments were performed in duplicate with a minimum of eight fields scored per well.

Assessment of β -cell death. Fetal cells were incubated for 20 min with the ERK-1/2 inhibitor U0126 (25 μ mol/l) or with DMSO vehicle alone. Cells and inhibitors were then added to four-well Permax chamber slides (5×10^4 cells per well) previously coated with vitronectin (75 nmol/l). After 2, 4, 8, 12, and 20 h, adherent cells were fixed with 4% paraformaldehyde, and apoptotic or dying cells were stained using a fluorescein-based in situ cell death detection kit (Roche Applied Science, Indianapolis, IN). These cells were then costained for insulin as previously described (3). The number of double-positive cells was enumerated using a fluorescent microscope. Multiple wells were seeded to allow the assessment of a minimum of 200 β -cells per treatment at each time point.

Analysis of insulin mRNA levels by real-time PCR. Fetal or adult cells were harvested and added to non-tissue culture-treated 48-well plastic plates previously coated with HTB-9, vitronectin, laminin-1, collagen IV, or BSA. Cells were added at 5×10^4 cells per well in fibroblast basal media adhesion buffer. Total RNA from nonadherent cells (BSA-blocked plastic) and from adherent cells (HTB-9, collagen IV, vitronectin, and laminin-1) was extracted after 1.5 and 24 h. cDNA was synthesized from 1 μ g total RNA using a SuperScript III RT kit (Invitrogen). Quantitative PCR was performed using TaqMan FAM dye-labeled MGB insulin and GAPDH probes (Applied Biosystems, Foster City, CA) and an Applied Biosystems Prism 7900HT sequence detection system (UCSD Cancer Center).

Data presentation and statistics. Data are the means \pm 1 SD. Statistical analysis was performed using Student's *t* test, and *P* values were based on data from multiple repeat experiments (*n* = 3–6).

RESULTS

Integrin-mediated adhesion and ERK activation promote insulin secretion by fetal β -cells. Fetal cells were added to wells precoated with common matrix constituents or the tumor-derived matrix HTB-9 (18), and insulin release was assessed after 90 min (Table 1). Poly-D-lysine, which supports β -cell attachment (but not integrin ligation), was used as an adhesion control.

β -Cells adhering to laminin-1, fibronectin, collagen I, collagen IV, vitronectin, and HTB-9 matrix all released significantly higher levels of insulin than cells on poly-D-lysine or BSA-blocked plastic (Table 1). This matrix-dependent release was observed using a basal media without the addition of exogenous growth factors, and vitronectin, collagen IV, and HTB-9 matrix were consistently found to be the most efficacious substrates (Table 1). Addition of serum and hepatocyte growth factor, which have been shown to promote optimal fetal islet-like cell cluster survival and growth (7,17), did not significantly impact insulin secretion induced by either collagen IV or HTB-9 matrix, but it did partially reduce insulin secretion

TABLE 1
Impact of different extracellular matrix constituents on insulin release

Substrate and media	Insulin release*	
	Percentage of content†	Fold increase‡
BSA (nonadherent)		
Basal§	1.1 ± 0.3	—
HGF/serum	1.2 ± 0.1	—
Poly-D-lysine (inert substrate)		
Basal	1.2 ± 0.2	1.1 ± 0.2
HTB-9 (tumor extracellular matrix)		
Basal	5.2 ± 0.3	4.7 ± 0.3
HGF/serum	5.8 ± 0.4	4.9 ± 0.3
Laminin-1 (basement membrane)		
Basal	2.9 ± 0.9	2.6 ± 0.8
Fibronectin (basement membrane)		
Basal	4.0 ± 1.0	3.6 ± 0.9
Collagen I (interstitial matrix)		
Basal	4.2 ± 0.8	3.8 ± 0.7
Collagen IV (basement membrane)		
Basal	6.3 ± 1.1	5.7 ± 1.0
HGF/serum	5.7 ± 0.1	4.8 ± 0.1
Vitronectin (extracellular matrix/serum)		
Basal	5.9 ± 1.1	5.4 ± 1.0
HGF/serum	2.4 ± 0.1	2.0 ± 0.1

Data are means ± SD. *Insulin release after 90 min on matrices or control substrates; †insulin release expressed as a percentage of the insulin content of cells added to the assay; ‡fold increase in insulin release relative to release by nonadherent cells maintained on BSA-blocked plastic; §fibroblast basal media without serum or growth factors; ||fibroblast basal media with HGF (10 ng/ml) and 10% FCS. HGF, hepatocyte growth factor.

on vitronectin (Table 1). Of note, wells coated with HTB-9 matrix, vitronectin, and collagen IV were further blocked with BSA to minimize the absorption of serum constituents that could influence adhesion (e.g., fibronectin or vitronectin). Under these experimental conditions, serum partially inhibited initial attachment to vitronectin (not shown) and consequently reduced short-term insulin secretion on this substrate (Table 1). Soluble vitronectin, prevented from binding to plastic by prior BSA blocking, was not found to induce insulin release (not shown). Fetal β -cells have been known to respond poorly to glucose stimulation (23), and varying glucose levels from 4 to 24 mmol/l had little impact on insulin secretion induced by collagen IV (not shown) or vitronectin (Fig. 1).

Insulin release resulting from adhesion to vitronectin and collagen IV was significantly inhibited by pharmacological inhibitors of the ERK pathway (U0126 or PD98059) (Table 2). In contrast, a control analog of U0126 (i.e., U0124) and inhibitors of other mitogen-activated protein kinases, including JNK (SP600125) and p38 (SB203850), had no significant effect (Table 2). To further confirm a role for the ERK pathway, fetal β -cells were infected with an adenovirus construct expressing a dominant-negative form of ERK together with GFP (Ad-DN-ERK). Infection with Ad-DN-ERK, but not with GFP alone (Ad-GFP), resulted in a significant reduction in insulin secretion on vitronectin (Table 2). Adenovirus infection did not significantly affect β -cell viability or adhesion to vitronectin.

We have reported that fetal β -cells use integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$ to adhere to vitronectin (3). To determine whether ligation of these integrins induces insulin secre-

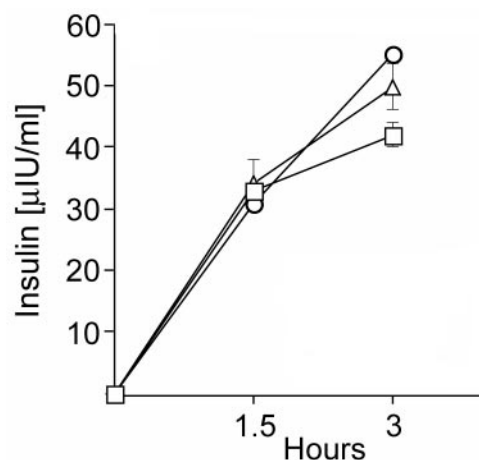


FIG. 1. Vitronectin-induced insulin secretion by fetal β -cells is independent of glucose levels. Fetal cells were plated on vitronectin in glucose-free Dulbecco's modified Eagle's medium supplemented with 4, 8, or 24 mmol/l glucose, and insulin release was determined after 1.5 and 3 h. ○, 24 mmol/l glucose; △, 8 mmol/l glucose; □, 4 mmol/l glucose.

tion, fetal β -cells were plated on vitronectin in the presence of function-blocking antibodies, and insulin secretion was assessed after 90 min. Inhibition of $\alpha_v\beta_5$ and both α_v - and β_1 -integrin subunits significantly reduced insulin secretion (Table 3). A modest but consistent reduction in insulin secretion was also observed after blocking $\alpha_v\beta_3$ (Table 3). These findings suggest that $\alpha_v\beta_5$ and $\alpha_v\beta_1$ are primarily responsible for inducing insulin secretion on vitronectin. We have previously demonstrated that $\alpha_1\beta_1$ induces insulin secretion on collagen IV (4).

Adhesion to vitronectin, collagen IV, and HTB-9 matrix induces a significant loss of insulin content in fetal β -cells. The long-term impact of adhesion on the insulin content of β -cells was assessed. Overnight attachment to vitronectin, collagen IV, and HTB-9 matrix in a basal media resulted in a significant decline in the insulin content of adherent β -cells (Fig. 2A). In contrast, the insulin content of nonadherent cells, maintained under

TABLE 2
Vitronectin and collagen IV induce insulin secretion via an ERK-dependent mechanism

Substrate and reagent	Inhibits	Insulin release* (% of control)†
Vitronectin		
DMSO	(Vehicle control)	100 ± 19
U0124	(Analog control)	87 ± 8
U0126	ERK-1/2	35 ± 7‡
PD98059	ERK-1/2	51 ± 8‡
SP600125	JNK	113 ± 31
SB203850	p38	112 ± 1
Vitronectin	(Uninfected control)	100 ± 12
Ad-GFP	(Virus control)	87 ± 10
Ad-DN-ERK	ERK-1/2	45 ± 6§
Collagen IV		
DMSO (vehicle)	(Vehicle control)	100 ± 4
U0126	ERK-1/2	45 ± 4‡

Data are means ± SD. *Insulin release after 90 min on vitronectin or collagen IV in the presence or absence of inhibitors or after infection with Ad-DN-ERK; †insulin release as a percentage of the secretion observed for control cells (DMSO vehicle alone or uninfected); ‡ $P < 0.05$ for treatment vs. DMSO control; § $P < 0.05$ for Ad-DN-ERK vs. virus control.

TABLE 3
Ligation of α_v integrins promotes insulin secretion on vitronectin

Antibody	Anti-	Insulin release (% of control)*
IgG control	—	100 ± 3
LM609	$\alpha_v\beta_3$	76 ± 4
P1F6	$\alpha_v\beta_5$	34 ± 3†
P4C10	β_1	42 ± 2†
VNR	α_v	41 ± 2†

Data are means ± SD. *Insulin release after 90 min on vitronectin in the presence or absence of function-blocking mAbs; insulin release is expressed as a percentage of the secretion observed for control cells treated with nonspecific IgG; † $P < 0.05$ for antibody treatment vs. IgG control.

identical experimental conditions, was largely sustained (Fig. 2A). Significant, but less marked, declines in the insulin content of adherent cells were also observed in the presence of serum and hepatocyte growth factor, and there was no detectable loss of insulin content from nonadherent cells under these media conditions (Fig. 2B).

Fetal β -cells attached to vitronectin in a basal media showed a progressive decline in insulin content, and this decline was accompanied by a reciprocal increase in the insulin content of the culture supernatant (Fig. 2C). The cumulative amount of insulin associated with nonadherent or detaching β -cells over the same time period was <8% (Fig. 2C). Simultaneous staining for insulin and fragmented DNA (transferase-mediated dUTP nick-end label staining) revealed that <10% of β -cells that initially attach to vitronectin in basal media undergo apoptosis or cell death during the assay period (Fig. 2D). Together, these data suggest that sustained insulin secretion, rather than β -cell death and detachment, is primarily responsible for the observed loss of insulin content after adhesion to vitronectin. Inhibition of ERK activation by U0126, while effectively inhibiting insulin secretion on vitronectin, had no effect on the rate of cell death (Fig. 2D).

The loss of insulin content resulting from adhesion to vitronectin in basal media (Fig. 2A) caused a corresponding decline in the number of adherent insulin-positive cells that could be detected by immunohistochemistry (Fig.

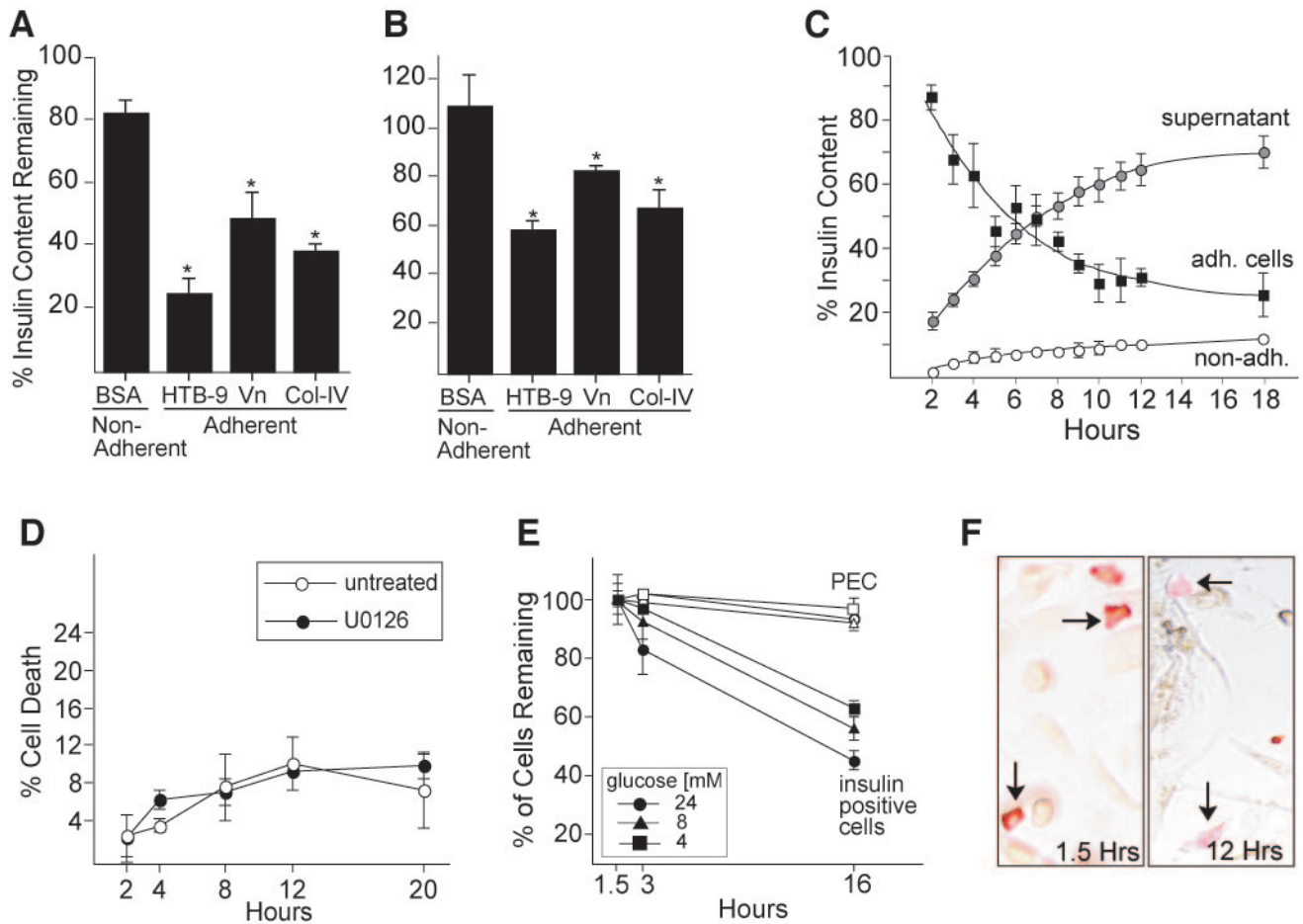


FIG. 2. Adhesion to vitronectin and collagen IV induces a significant loss of insulin content in fetal β -cells. **A** and **B**: Fetal cells were seeded onto HTB-9 matrix, vitronectin (Vn), or collagen IV (Col-IV) in a basal media (fibroblast basal media) without (**A**) or with (**B**) serum and hepatocyte growth factor. The insulin content of adherent (adh) and nonadherent (non-adh) cells (BSA) was assessed after 1.5 and 18 h. Results show the percentage of insulin remaining after 18 h. * $P < 0.05$ for adherent cells vs. nonadherent. **C**: Fetal cells were seeded onto vitronectin in a basal media (fibroblast basal media), and the amount of insulin released into the supernatant was assessed over 2–18 h. The insulin content of β -cells attached to the substrate (adherent) or detaching over time (nonadherent) was also determined. Results show the percentage of insulin present in each fraction. **D**: Fetal cells were added to slides coated with vitronectin in a basal media (fibroblast basal media) in the presence of U0126 or DMSO vehicle alone. After 2–20 h, cells were stained for insulin and fragmented DNA. Results show the percentage of insulin-positive cells undergoing cell death. **E**: Fetal cells were seeded onto vitronectin in the presence of 4, 8, or 24 mmol/l glucose, and adherent insulin-positive cells and pancreatic epithelial cells (PEC) were counted after 1.5, 3, and 16 h. Results show the percentage of cells remaining at 3 and 16 h. **F**: Photomicrographs (40 \times) showing insulin-positive cells 1.5 and 12 h after adhesion to vitronectin.

TABLE 4
Inhibition of ERK activation preserves the insulin content of adherent β -cells

Substrate and reagent	Inhibits	Insulin-positive cells (% remaining)*	Insulin content (% remaining)†
Vitronectin			
DMSO (vehicle)	—	53 \pm 7	48 \pm 9
U0124	(Analog control)	51 \pm 2	Not tested
U0126	ERK-1/2	92 \pm 10‡	72 \pm 9‡
SP600125	JNK	40 \pm 7	Not tested
SB203850	p38	26 \pm 6	Not tested
Vitronectin	(Noninfected)	49 \pm 12	Not tested
Ad-GFP	(Virus control)	50 \pm 2	Not tested
Ad-DN-ERK	ERK-1/2	77 \pm 2§	Not tested
Collagen IV			
DMSO	—	31 \pm 5	38 \pm 2
U0126	ERK-1/2	73 \pm 10‡	64 \pm 9‡

Data are means \pm SD. *Adherent insulin-positive cells were counted after 1.5 or 18 h for each treatment. Results show the percentage of cells present at 1.5 h that remain after 18 h. †The insulin content of adherent cells was determined after 1.5 or 18 h in the presence or absence of U0126. Results show the percentage of insulin content present at 1.5 h that remains after 18 h. ‡ $P < 0.05$ for U0126 treatment vs. DMSO control. § $P < 0.05$ for Ad-DN-ERK vs. virus control.

2E). This decline was only marginally increased at high glucose levels (i.e., 24 mmol/l), and epithelial cells (pancreatic epithelial cells), present in the same cell preparations, remained attached and viable throughout the assay (Fig. 2E). In addition to a decline in the number of insulin-positive cells, the staining intensity of those insulin-positive cells remaining on vitronectin after 12 h was significantly reduced (Fig. 2F). These weakly staining cells were still viable because they remained attached and spread on the substrate (Fig. 2F).

Inhibition of the ERK pathway preserves the insulin content of adherent fetal β -cells. Experiments were performed to determine whether ERK-dependent insulin secretion contributes to the loss of insulin content observed in adherent cells. Addition of the ERK inhibitor U0126 significantly ameliorated the loss of insulin content observed on both collagen IV and vitronectin and helped to maintain the number of insulin-positive cells adhering to these substrates (Table 4). Infection with the dominant-negative construct Ad-DN-ERK (21,22) also limited the loss of insulin-positive cells (Table 4). The preservation of insulin content observed in the presence of U0126 cannot be attributed to the inhibition of cell death because this inhibitor had no impact on the levels of β -cell apoptosis or cell death observed on vitronectin (Fig. 2D). Together, our data suggests that adhesion to vitronectin and collagen IV induces a marked decline in insulin content by inducing protracted ERK-mediated insulin secretion.

Collagen IV, but not vitronectin, induces ERK-dependent insulin secretion in adult β -cells. Studies were performed to determine whether adhesion to collagen IV and vitronectin induces comparable responses in adult β -cells. The impact of adhesion on both short-term insulin release and on insulin content was assessed, and the adult cells were tested in a serum-free media containing basal levels of glucose (2.8 mmol/l). Adhesion of adult cells to collagen IV resulted in a 300% increase in insulin release relative to cells on poly-D-lysine or BSA-blocked plastic and resulted in a 40% decline in insulin content after 18 h (Table 5). As seen in fetal cells, addition of the ERK inhibitor U0126 effectively inhibited insulin release on collagen IV and preserved insulin content (Table 5). Under identical experimental conditions, vitronectin failed to induce insulin secretion and had no significant impact on

insulin content (Table 5). This result cannot be attributed to a failure of adhesion because adult and fetal β -cells adhere equally to vitronectin (3).

Fetal and adult β -cell adhesion to vitronectin and collagen IV significantly suppresses insulin gene transcription. The impact of adhesion on insulin gene transcription was assessed. Fetal or adult β -cells were maintained in suspension on BSA-blocked plastic or were allowed to adhere to vitronectin, collagen IV, or laminin-1 (entactin-free). After 2 and 24 h, adherent cells were harvested and assessed for insulin mRNA by real-time PCR. Adhesion of fetal and adult β -cells to both vitronectin and collagen IV resulted in a significant decline in insulin mRNA levels after 24 h (Fig. 3A and B). Under identical experimental conditions, insulin mRNA levels in nonadherent cells (BSA-blocked plastic) were largely maintained or were increased, and attachment to laminin-1 had little or no impact (Fig. 3A and B). Addition of hepatocyte growth factor and serum to optimize the survival of fetal β -cells (7,17) did not prevent these cells

TABLE 5
Impact of vitronectin and collagen IV on insulin release and content in adult β -cells

Substrate and inhibitor	Release (fold increase)*	Content (% remaining)†
BSA	—	81 \pm 8
Poly-D-lysine	1.1 \pm 0.2	Not tested
Collagen IV	3.2 \pm 0.5	59 \pm 2
U0126	1.3 \pm 0.1‡	78 \pm 14‡
Vitronectin	1.2 \pm 0.2	87 \pm 4

Data are means \pm SD. *Insulin release after 90 min on collagen IV, vitronectin, or poly-D-lysine. Results show fold increase in insulin release relative to nonadherent cells on BSA-blocked plastic. †The insulin content of adherent cells was determined after 1.5 or 18 h in the presence or absence of U0126. Results show the percentage of insulin content present at 1.5 h that remains after 18 h. ‡ $P < 0.05$ for U0126 treatment vs. DMSO control.

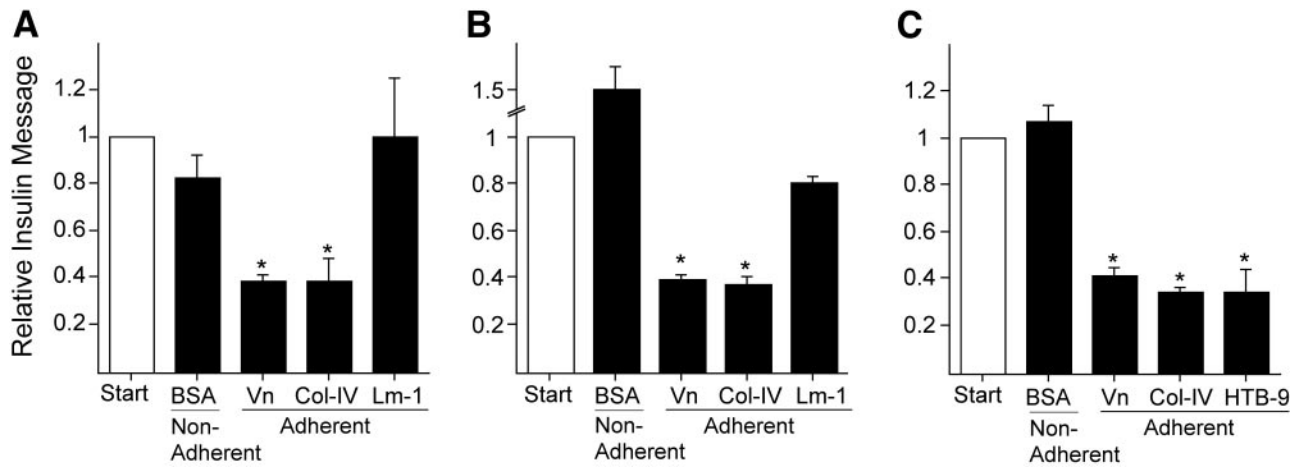


FIG. 3. Adhesion to vitronectin and collagen IV suppresses insulin gene transcription. *A* and *B*: Fetal (*A*) or adult cells (*B*) were seeded onto vitronectin (Vn), collagen IV (Col-IV), laminin-1 (Lm-1), or BSA-blocked plastic in a basal media (fibroblast basal media). Total RNA was extracted from adherent and nonadherent cells (BSA) after 1.5 and 24 h, and levels of insulin mRNA were assessed by real-time PCR. Results show the relative amount of insulin mRNA remaining after 24 h. *C*: Fetal cells were seeded onto vitronectin, collagen IV, and HTB-9 matrix in the presence of serum and hepatocyte growth factor and relative insulin mRNA levels determined as described above. All samples were normalized against GAPDH. * $P < 0.05$ for vitronectin, collagen IV, and HTB-9 matrix vs. nonadherent control.

from losing insulin message on vitronectin, collagen IV, or HTB-9 matrix (Fig. 3C). Together, these findings indicate that the observed loss of insulin message is caused by adhesion to select substrates and is not the result of adverse experimental conditions.

DISCUSSION

We have shown that the adhesion of human fetal β -cells to a variety of matrix constituents results in enhanced insulin secretion. Two of these constituents, vitronectin and collagen IV, induced the highest levels of secretion, and this secretion was shown to be both ERK and integrin dependent. The matrix-induced insulin secretion observed was glucose independent and appeared to be unregulated because it ultimately resulted in a significant depletion of insulin content. Additional studies using adult islets showed that mature β -cells also lose insulin content on collagen IV, but they are unaffected by vitronectin. Using real-time PCR, we have shown that adhesion of fetal and adult β -cells to select substrates (vitronectin, collagen IV, and HTB-9 matrix) also significantly suppresses insulin gene transcription. The loss of insulin production and content described in this study was not found to be associated with cell death.

Based on our findings, a variety of integrin-matrix interactions would have to be tightly regulated within islets to prevent a loss of insulin production or content. Strict regulation of integrin expression or matrix deposition may serve this purpose. Collagen IV is present in the basement membranes of intravascular blood vessels (4,24), and β -cells have been shown to reside in close apposition to these structures (25). However, the expression of collagen-binding integrins within developing islets is highly restricted (4). In this regard, we have shown that integrin $\alpha_1\beta_1$ promotes insulin secretion on collagen IV, but expression of this integrin is limited to a very small subset of β -cells in fetal islets (4).

Vitronectin has been described in pancreatic epithelia, but it is largely absent from islets (26). Although vitronectin-binding α_v integrins have been detected in developing islets, their expression in these structures is markedly lower than that seen in adjacent epithelia (26). In contrast

to their fetal counterparts, adult β -cells did not secrete insulin in response to vitronectin and did not lose insulin content on this substrate. In this regard, we have previously shown that adult β -cells, unlike fetal β -cells, do not express or utilize integrin $\alpha_v\beta_1$ (3). This is significant because $\alpha_v\beta_1$, in conjunction with $\alpha_v\beta_5$, appears to have an essential role in inducing insulin secretion by fetal β -cells. Evidently, a lack of $\alpha_v\beta_1$ expression in adult β -cells could account for their failure to respond to vitronectin and may help to preserve the insulin content of mature islets.

Environmental factors that serve to restrict vitronectin or collagen IV interactions within islets are likely to be lost during ex vivo culture. We have shown that $\alpha_1\beta_1$ expression is strongly induced after isolation and culture of fetal and adult β -cells (4), and this will increase the potential for collagen binding. β -Cells in culture will be exposed to collagens deposited by contaminating fibroblasts or epithelial cells, and culture in the presence of serum will inevitably result in exposure to vitronectin. Originally described as serum-spreading factor, vitronectin is known to be absorbed from serum onto tissue culture plastic, where it supports significant cell adhesion and spreading.

HTB-9 matrix and other complex matrices, in conjunction with hepatocyte growth factor and serum, have been shown to provide an optimal environment for the proliferation of fetal islet-like cell clusters (7,17). However, this proliferation is also associated with a reciprocal decline in insulin message and content (7,17). Our findings suggest that much of this decline may be attributed to direct integrin-dependent adhesion to the underlying matrix. Interestingly, removing proliferating cells from such matrices and reaggregating them restored insulin expression (17). Evidently, adhesion and expansion on these matrices does not cause an irreversible loss of fetal β -cells but may promote dedifferentiation and subsequent anchorage-dependent growth. Recently, it has been shown that β -cells lose insulin and proliferate in a process akin to epithelial-to-mesenchymal transition (27). In this regard, both integrins and the extracellular matrix are known to play an essential role in promoting epithelial-to-mesenchymal transition (28,29).

Fetal islet-like cell clusters grown on HTB-9 matrix in

the presence of hepatocyte growth factor have been shown to lose insulin content over a period of 7 days (7). This loss of insulin content contrasts with the more precipitous declines described in this study. However, the timing and extent of contact between the fetal β -cells and the HTB-9 matrix may account for this disparity. In our study, we utilized single-cell suspensions, which allows β -cells to attach directly to the matrix within 60–90 min. Intact islet-like cell clusters take several days on HTB-9 matrix to transition into expanding monolayers. During this transition, most of the β -cells are involved in cell-cell interactions and may have little direct contact with the underlying matrix.

In this study, we have shown that adhesion to laminin-1 does not induce a significant loss of insulin message. This is important because it suggests that not all matrix interactions have a detrimental impact on insulin production. Some reports have shown that β -cells cultured in three-dimensional gels, such as matrigel or fibrin, are able to maintain insulin expression (14–16). These compliant gels permit β -cells to be cultured within large cellular aggregates (e.g., islet-like cell clusters). Cell-cell, rather than cell-matrix, interactions are likely to dominate such cultures, and this may alter both the extent of integrin ligation and the nature of concomitant signaling events.

Results presented in this article have important implications for the ex vivo expansion of β -cells for transplantation. During ex vivo culture, β -cells are inevitably exposed to matrix constituents that have either been added to promote survival and growth or have been deposited by pancreatic cells themselves. For the first time, we have identified specific matrix interactions and signaling events that have a profound impact on the secretion, storage, and transcription of insulin by cultured human β -cells. Ultimately, circumventing or blocking these interactions and signaling events could help to optimize or restore the functionality of β -cells during ex vivo culture.

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