Alpha<sub>v</sub>-integrin utilization in human β-cell adhesion, spreading and motility<sup>*</sup>

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SUMMARY

The role of individual integrins in human β-cell development and function is largely unknown. This study describes the contribution of αv-integrins to human β-cell adhesion, spreading and motility. Developmental differences in αv-integrin utilization are addressed by comparing the responses of adult and fetal β-cells and vitronectin is used as a substrate based on its unique pattern of expression in the developing pancreas. Fetal and adult β-cells attached equally to vitronectin and integrin αvβ5 was found to support the adhesion of both mature and immature β-cell populations. Fetal β-cells were also observed to spread and migrate on vitronectin, and integrin αvβ1 was found to be essential for these responses. In contrast to their fetal counterparts, adult β-cells failed to either spread or migrate and this deficit was associated with a marked down regulation of αvβ1 expression in adult islet preparations. The integrin αvβ3 was not found to support significant β-cell attachment or migration. Based on our findings, we conclude that integrins αvβ5 and αvβ1 are important mediators of human β-cell adhesion and motility, respectively. By supporting fetal β-cell migration, αvβ1 could play an important role in early motile processes required for islet neogenesis.
INTRODUCTION

Integrins are a family of heterodimeric transmembrane adhesion molecules comprised of non-covalently associated α- and β-subunits. By external ligand recognition and concomitant recruitment of cytoskeletal and signaling elements these recognition molecules serve to integrate a cell's interior machinery with the extracellular environment (1). Integrins have been implicated in a plethora of processes required for normal development including cell survival, proliferation, cytodifferentiation, migration and spatial segregation (1-6).

The αv-integrin subfamily currently contains five members (αvβ3, αvβ5, αvβ1, αvβ6 and αvβ8) which all recognize components of the extracellular matrix (ECM) via the linear tri-peptide sequence arg-gly-asp (RGD). Despite recognizing a common epitope there are marked differences in ligand binding specificity of these αv-integrins (7). The different αv-integrins also have unique spatial and temporal patterns of cell and tissue expression during organogenesis implying different and potentially important roles in development (8). Thus far either animal or in vitro studies have indicated a role for αv-integrins in embryo implantation (9,10), metanepheric development (11), vasculogenesis (9,12), angiogenesis (13), bone remodeling (14) and palate formation (9).

Among the various ECM-components recognized by αv-integrins, vitronectin (VN) has the distinction of being recognized by all five of the known αv-subfamily members (15). Following ECM-sequestration, VN can regulate a variety of cellular processes including migration, differentiation, proliferation and morphogenesis (15,16). Importantly, VN expression has recently been confirmed in the developing human pancreas in association with both epithelial cells and insulin-expressing cells emerging from the ductal epithelium (17). Based on this highly restricted distribution it is suggested that endocrine progenitors may arise from a larger pool of VN-producing cells (17). An important inference of this study is that local VN biosynthesis and

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1 The abbreviations used are: ECM, extracellular matrix; PEC, pancreatic epithelial cells; VN, vitronectin; pAb, polyclonal antibody; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting.
deposition could instigate or otherwise modulate critical migratory or morphogenic events required for islet neogenesis.

Studies assessing the impact of matrix interactions on β-cell development and function are limited in number, but provide important evidence that the matrix can influence many aspects of β-cell biology including survival (18,19), proliferation (20, 21), differentiation (22, 23), and insulin secretion (24). Interaction with ECM has been shown to be important for the long-term ex-vivo survival of β-cells (18) and collagen, matrigel, and fibrin gels have been shown to provide a compliant matrix environment suitable for maintaining islet viability and function (25, 26, 27). Complex laminin-rich matrices have also been shown to facilitate islet formation (28), to promote the proliferation of human β-cells in response to hepatocyte growth factor (20), and to amplify insulin-secretion in response to secretagogues (24). As a purified ligand, laminin, has been shown to promote the differentiation of fetal mouse pancreatic β-cells (22, 23). In most cases the requisite integrins involved in these responses remains to be determined.

The contribution of α₅-integrins to β-cell development and function has not been assessed. However, α₅β₃ and α₅β₅ expression has been described in the developing human pancreas both on ductal epithelium and in immature islets and both of these integrins are proposed to support pancreatic epithelial cell (PEC) adhesion (17). Importantly, a cyclic RGD peptide, that can disrupt α₅-integrin ligation, has also been shown to prevent normal islet development in vivo (17).

In this study we assess, for the first time, the impact of α₅-integrin expression and utilization on human β-cell adhesion, spreading, and migration. VN is used as the primary substrate based on its ability to support interactions with multiple α₅-integrins and its unique pattern of expression in the developing pancreas (15, 17). Our studies identify those α₅-integrin heterodimers required for β-cell adhesion, spreading and migration and contrast the α₅-integrin mediated responses of both adult and fetal β-cells. These studies highlight significant developmental differences in α₅-integrin mediated motility between fetal and adult β-cells and indicate a potentially important role for α₅β₁ in motile processes required for early islet neogenesis.
MATERIALS AND METHODS

Reagents — Function-blocking monoclonal antibodies (mAbs) to human $\alpha_v\beta_3$ (LM609), $\alpha_v\beta_5$ (PIF6) and the $\beta_1$-subunit (P4C10) were obtained from Chemicon (Temecula, CA). A blocking mAb to the human $\alpha_c$-subunit (L1A3) was kindly provided by Dr A. Strongin (Burnham Institute, La Jolla). An anti-$\alpha_v$ polyclonal antibody (pAB; VNR) with potent function-blocking activity to $\alpha_v$-integrins (29) was generated at the Scripps Research Institute (La Jolla, CA). MAbs used for western blotting including anti-human $\alpha_v$ (LM142), $\beta_1$ (LM534) and $\beta_3$ (AP3) were purchased from Chemicon. A mAb to human insulin (2D11-H5) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA) and a sheep pAb (PC059) to insulin was from The Binding Site Inc. (San Diego, CA). Secondary donkey anti-sheep and anti-mouse immunoglobulin antibodies conjugated to alkaline phosphotase or fluorescein were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified human VN was from Chemicon (Temecula, CA).

Tissue procurement, processing and cell culture — Institutional approval was obtained for the use of fetal and adult human pancreata. Human adult cadaveric pancreata (ages 37-65 years) were obtained via the JDRF Human Islet Distribution Program and human fetal pancreata (19-21 weeks gestational age) were provided by Advanced BioResources (Alameda, CA).

Fetal pancreata were digested with a collagenase solution (Collagenase-P; 2.5 mg/ml) as described (20). Resulting cell clusters were re-suspended in RPMI-1640 containing 10% normal human serum and were incubated overnight to allow the formation of islet-like cell clusters (ICCs) (20). These ICCs typically contained 75-85% epithelial cells and 2-4% insulin-reactive cells (30). ICCs derived from individual pancreases (approximately 100-200) were then seeded into Petri dishes coated with a HTB-9 matrix and were then grown as monolayers in the presence of RPMI-1640, 10% FBS, and 10 ng/ml of hepatocyte growth factor (HGF; Genentech, San Francisco, CA) as described (20). Cells were expanded for 3 days prior to use and were confirmed to contain 85-90% pancreatic epithelial cells (PEC) and 1-2% insulin-positive $\beta$-cells.
Human islet preparations were isolated in-house using a modified semi-automated method essentially as described (31). Islet clusters were subsequently expanded on HTB-9 matrix for 3-5 days prior to use as described for fetal ICC preparations. Expanded adult islet preparations typically contained 30-40% insulin-positive β-cells and 50-60% epithelial cells.

**Immunofluorescence and morphological analysis** — The morphology and actin organization of adherent β-cells was assessed by double staining for insulin and F-actin. Adult and fetal pancreatic cell monolayers grown on HTB-9 matrix were harvested with a 0.025% trypsin/Versene solution (Versene:1:5000; Invitrogen), and single cell populations were added to glass chamber slides (Nalge Nunc, Naperville, IL) previously coated with VN (5 μg/ml for 4 hours at 37°C). After 90 minutes, adherent cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton-X in PBS for 10 minutes. After blocking (2% donkey serum and 1% BSA in PBS) the cells were incubated with an anti-insulin mAb (2D11-H5 at 1:250; Santa Cruz) for 45 minutes. The cells were subsequently incubated for 45 minutes with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (Jackson). To reveal F-actin organization the cells were washed and further stained for 20 minutes with an Alexa Fluor 546 phalloidin conjugate (1:100 dilution; Molecular Probes, Eugene, OR). Cells were photographed using an inverted Nikon Eclipse E800 fluorescent microscope and a 60X objective.

**Cell adhesion and spreading assays** — 96-well high binding EIA plates (Costar, Corning, NY) were coated with VN (5 μg/ml in PBS) for 4 hours at 37°C. These wells were subsequently blocked with 5% BSA for 45 minutes prior to use. Control wells received BSA alone. Adult and fetal pancreatic cell monolayers on HTB-9 matrix (70-80% confluence) were harvested with a 0.025% trypsin/Versene solution and single cell populations were added at 1 X 10⁴ cells/well in fibroblast basal media (FBM; BioWhittaker) supplemented with 0.5% BSA and 0.4mM MnCl₂ (pH 7.4). The contribution of integrins to cell adhesion and spreading was assessed using function-blocking antibodies as specified in the text. The cells were pretreated with the antibodies for 20 minutes prior to the addition of both cells and antibodies to VN-coated wells. Anti-integrin mAbs were
used at 40μg/ml. Cells were allowed to adhere at 37°C for 90 minutes and were then carefully washed and non-adherent cells removed under a constant vacuum. Remaining adherent cells were fixed with 3.7% paraformaldehyde (PFA).

To detect adherent β-cells, the PFA-fixed cells were permeabilized with 0.1% Triton-X, blocked, and incubated for 45 minutes with a sheep pAb to human insulin (dilution 1:500; The Binding Site Inc.). After washing, the cells were incubated for a further 45 minutes with an alkaline phosphatase-conjugated donkey anti-sheep antibody absorbed against mouse serum (dilution 1:250; Jackson). Color was developed using Vector Red® substrate as recommended by the manufacturer (Vector Labs, Burlingame, CA). Insulin-positive cells per field were counted using an inverted microscope equipped with a 20x objective and an ocular grid. β-cell spreading was assessed by enumerating round versus spread insulin-positive cells per field. Treatments were performed in duplicate and a minimum of 8 fields were scored per well. After evaluating β-cell adhesion adherent epithelial cells were visualized by staining with 1% toluidine blue for 20 minutes. The adhesion and spreading of these cells was assessed as described for β-cells. The percentage of β-cells attaching to immobilized VN was determined by calculating the number of insulin positive cells seeded per well versus the number of positive cells that had attached per well by the end of the assay. The number of positive cells seeded was determined by immunostaining harvested cells in suspension and then counting positive cells with the aid of a haemocytometer.

Migration Assays — Migration was assessed using 6-well Transwell migration plates from Costar. The undersides of the insert membranes (8.0 μm pore size) were coated with VN (5μg/ml in PBS) for 4 hrs at 37°C. Control wells were treated with 5% BSA. Adult and fetal pancreatic cells expanded on HTB-9 matrix (70-80% confluence) were harvested with 0.025% trypsin/Versene and were re-suspended in FBM supplemented with 0.5% BSA and 0.4mM MnCl₂ (pH 7.4). The cells were then added to migration plates at 5 x 10⁵ cells per insert. The contribution of integrins to migration was assessed using function-blocking antibodies as specified in the results section. The cells were pretreated with the antibodies for 20 minutes prior to the addition of both cells and antibodies to migration plates. Anti-integrin mAbs were used at 40μg/ml and were added to upper
and lower chambers. Directed migration from the upper to lower chamber of the transwell was
determined after 12-14 hours.

Cells on the underside of the membrane inserts were fixed, permeabilized and stained for
insulin as described above for adhesion and spreading assays. The upper side of the membrane
inserts was swabbed to remove cells that failed to migrate. The entire underside of the insert was
then scanned and counted for insulin-positive cells using an inverted microscope equipped with a
40x objective. Migrant epithelial cells on the underside of the inserts were counted after staining
with 1% Toluidine blue. Epithelial cells were counted per grid field using a 20x objective and a
minimum of 8 fields were scored per membrane.

**Immunoprecipitation and western blot analysis** — The integrin expression profiles of adult and
fetal pancreatic cells were determined by immunoprecipitation and western blot analysis. Fetal and
adult pancreatic preparations were expanded for 3 days on HTB-9 matrix prior to the harvesting of
cell monolayers using a lysis buffer containing 150 mM NaCl, 50 mM TRIS, 1% NP40 (pH 8.0)
and an EDTA-free protease inhibitor cocktail (Roche). After 30 minutes on ice, lysates were
clarified by centrifugation at 14,000X g for 20 minutes and were pre-cleared by mixing with
protein-G agarose beads (100μl beads/500μg of lysate; Pierce) for 2 hours at 4°C. Cleared lysates
were then incubated overnight at 4°C with a rabbit pAb to the αv-integrin subunit (1:100 dilution;
AB1930; Chemicon). An equal quantity of protein was also incubated with 5μl normal rabbit
serum as a control. Integrin-antibody complexes were immunoprecipitated by mixing with protein-G
Agarose beads (100μl) for 3 hours at 4°C. The beads were then washed, mixed in non-reducing
sample buffer and boiled for 5 minutes. Immunoprecipitates and untreated cell lysates were
electrophoresed under non-reducing conditions on 5-12% Tris-Glycine polyacrilamide gels.
Protein was transferred to PVDF paper and blotted with antibodies to various integrin subunits
including β1 (LM534), β3 (AP3), β5 (11D1), and αv (LM142).

**Flow cytometry** — Surface integrin expression associated with fetal or adult pancreatic cells was
assessed by flow cytometry (FACS) analysis. Sub-confluent monolayer cultures were harvested
with 0.025% trypsin/Versene, washed and stained with FITC-conjugated antibodies to human $\alpha_v$ (IMM-1044; Immunotech), $\alpha_v\beta_3$ (PM6/13; Chemicon), and $\alpha_v\beta_5$ (P1F6; Chemicon). FITC-conjugated isotype matched antibodies were used as controls. Expression of the $\beta_1$-subunit was determined by staining a mAb P4C10 (Chemicon) followed by a FITC-labeled donkey anti-mouse IgG secondary (Jackson). Cells were incubated with primary or secondary antibodies for 60 minutes in 100 $\mu$l of ice cold FACS buffer (PBS, 0.1%BSA, 0.2% sodium azide). Cells were analyzed using a FACscan flow cytometer (Becton Dickinson; Mountain View, CA).

RESULTS

Fetal and adult $\beta$-cells adhere equally to VN but show disparate levels of spreading and actin organization — VN was selected as a substrate for adhesion assays based on its expression in the developing pancreas and its ability to serve as ligand for multiple $\alpha_v$-integrins. Levels of $\beta$-cell and pancreatic epithelial cell (PEC) attachment over time were assessed using both fetal and adult pancreatic preparations. The number of insulin positive cells plated at the start of the assay was determined by immunostaining and then counting positive cells with a haemocytometer. The percentage of $\beta$-cells adhering to immobilized VN was then calculated by staining and counting the number of attached insulin positive cells per well. Using this approach, it was determined that approximately 50% of the $\beta$-cells plated attached to VN over a 30-180 minute time course (Fig. 1A). Levels of attachment between adult and fetal $\beta$-cells were comparable (Fig. 1A) and no attachment to BSA-blocked control wells was observed (not shown). Adult and fetal PEC attachment was also equivalent, but the fraction of PEC adhering to VN was higher than that observed for both adult or fetal $\beta$-cells (Fig. 1A). Please note, that only strongly adherent $\beta$-cells are likely to remain after the multiple washing steps required for insulin immunostaining.

Despite comparable levels of adhesion, adult and fetal $\beta$-cells showed marked differences in their ability to spread and reorganize actin after attachment to VN. While the majority of fetal $\beta$-cells spread after 90 minutes, most adult $\beta$-cells failed to do so (Fig. 1B; left panel). Despite the spreading deficit of adult $\beta$-cells, adult PEC in the same preparations spread just as well as their
fetal counterparts (Fig. 1B, right panel). The disparity in spreading between insulin positive fetal and adult cells is shown in figures 2 A and B. By simultaneous staining for insulin (green) and actin (red) it was confirmed that fetal β-cells reorganize their actin to form small actin-rich filopodia and weak stress fibers while most adult β-cells (>80%) had no clear actin organization whatsoever (Fig. 2C-D). In contrast to fetal and adult β-cells, PEC in the same preparations adopted a characteristic epithelial morphology with large well-defined lamellipodia and distinct stress fibers (Fig. 2C-F). Occasional adherent fibroblasts (Fb.) in the pancreatic preparations were distinguished by a characteristic elongated morphology (Fig. 2E).

These findings confirm that VN can serve as a robust substrate for β-cell attachment and point to a profound developmental difference in the relative ability of adult and fetal β-cells to spread and reorganize actin on this substate.

β-cells utilize multiple integrins to attach to VN but require αvβ1 for a spreading response —

Adhesion assays were performed using fetal ICC and adult islet preparations and the contribution of individual integrin subunits or heterodimers assessed using specific function blocking antibodies. β-cell adhesion to VN in the presence or absence of these antibodies was assessed by counting the number of adherent insulin-positive cells per unit area.

Confirming a dominant role for αv-integrins, a function blocking antibody to the αv-subunit blocked adult and fetal β-cell adhesion by approximately 70% (Figs. 3A and B). Using an antibody specific for the αvβ5 heterodimer it was confirmed that 50-60% of this αv-mediated adhesion can be attributed to ligation of αvβ5 (Fig. 3A and B). Blocking the β1-integrin subunit also had a significant impact, particularly on the adhesion of fetal β-cells (Fig. 3A and B). β1-integrins known to recognize VN, and therefore candidates for supporting β-cell adhesion, include both αvβ1 and α8β1. A further determination of the potential contribution of each of these integrins is, however, complicated by a lack of function-blocking antibodies specific for these heterodimers. The contribution of integrin αvβ3 was consistently found to be minor or insignificant (Fig. 3A and B). An evaluation of PEC adhesion within the same assays confirmed that these cells, like β-cells, have a strong dependence on αv-integrins and αvβ5 in particular (Fig. 3C and D).
Fetal β-cells that still attached to VN in the presence of antibodies to the αv- or β1-integrin subunit were almost entirely prevented from spreading (Fig. 3E). Complete abrogation of function as a result of blocking either αv- or β1-subunits is regarded as an indication of αvβ1 involvement (32). Accordingly, the results presented in figure 3E can be fully explained on the basis that integrin αvβ1 makes a significant contribution to fetal β-cell spreading. Importantly, expression of the αvβ1 heterodimer by fetal pancreatic cells was confirmed by immunoprecipitation studies, which are described later in this report.

Significant β-cell spreading still occurred in the presence of an antibody to αvβ5 despite the important contribution of this integrin to initial β-cell attachment (Fig. 3E). Accordingly, αvβ5 either fails to support significant β-cell spreading or is not essential for this process. The finding that blocking β1 completely prevents β-cell spreading supports the notion that αvβ5 cannot substitute for αvβ1 in this process. However, it is important to note that in contrast to fetal β-cells, fetal PEC were able to use both αvβ1 and αvβ5 for cell spreading (Fig. 3F).

β-cell migration to VN is only evident in fetal cells and requires αvβ1 — Conventional transwell migration assays were modified to allow the detection of insulin-positive cells that had migrated to VN. Migration assays were performed using fetal and adult pancreatic preparations, and migrant β-cells were detected on the underside of porous membrane inserts by staining for insulin (Fig 4A). Subsequent staining with toluidine blue allowed the further detection of migrant PEC and occasional fibroblasts (Fb.) (Fig. 4B).

Although fetal and adult β-cells adhered equally well to VN (Fig. 1A), only fetal β-cells were able to migrate to this substrate (Fig. 5A). The migration deficit of adult β-cells was not reciprocated by adult PEC, which migrated just as well as their fetal counterparts (Fig. 5B). Indicating an important role for αvβ1, the migration of fetal β-cells to VN was completely abrogated in the presence of antibodies to either the αv- or β1-integrin subunit (Fig. 5C). This result is consistent with the finding that αvβ1 is also required for fetal β-cell spreading (Fig. 3E), a process which necessarily precedes haptotactic migration. An antibody to αvβ5 also reduced fetal β-cell migration by approximately 50% (Fig. 5C). However, it is evident that αvβ5 alone cannot...
support β-cell migration since there is no significant migration after blocking β₁-ligation (Fig. 5C). Furthermore, despite using αᵥβ₅ for adhesion, adult β-cells are clearly unable to migrate using this integrin (Fig. 5A). Accordingly, the antibody to αᵥβ₅ may inhibit fetal β-cell migration by disrupting initial attachment (Fig. 3A) rather than motility.

The migration of fetal PEC to VN was also found to be entirely dependent on αᵥ-integrins, however, there was a less stringent requirement for β₁ (Fig. 5D). This result suggests that in contrast to fetal β-cells, fetal PEC may use αᵥβ₅ alone for motility responses. Such a result is consistent with the prior observation that αᵥβ₅ contributes more to PEC spreading than to β-cell spreading (Fig. 3E and F).

Expression of αᵥβ₁ is developmentally regulated — Relative levels of αᵥ-integrin expression in fetal or adult pancreatic preparations were determined by immunoprecipitation with an anti-αᵥ antibody followed by western blot analysis to detect β₅, β₁, and β₃ subunits. Using this approach, high levels of αᵥβ₁ and αᵥβ₅, but low levels of αᵥβ₃ were confirmed in fetal pancreatic preparations (Fig. 6A; left panel). Importantly, this pattern of expression is consistent with the relative contribution of these integrins to the adhesion and migration of fetal β-cells and PEC (Figs. 3 and 5).

Expression of αᵥβ₁ was markedly reduced in adult pancreatic preparations while expression of both αᵥβ₃ and αᵥβ₅ remained largely unchanged (Fig. 6A; right panel). Such a marked reduction in αᵥβ₁ expression was noted irrespective of whether the αᵥ-subunit was immunoprecipitated from detergent-soluble or -insoluble protein fractions (not shown). This result suggests that αᵥβ₁ expression is strongly down regulated during the course of pancreatic development. Such a developmental loss of αᵥβ₁ expression provides one explanation for the inability of adult β-cells to spread or migrate on VN (Figs. 3 and 5) and could represent an important mechanism for regulating β-cell motility during islet neogenesis. The reduction in αᵥβ₁ expression observed cannot simply be attributed to a reduction in the overall expression of β₁- or αᵥ-subunits since fetal and adult lysates had comparable levels of these subunits (Fig. 6B).
Levels of $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v$ or $\beta_1$ on the surface of fetal or adult pancreatic cells was also assessed by FACS analysis (Fig. 6C). This approach confirmed that $\alpha_v\beta_5$ is expressed at higher levels than $\alpha_v\beta_3$, and confirmed that expression of $\alpha_v\beta_5$ and $\alpha_v$ and $\beta_1$-subunits is maintained in adult islet preparations. The lack of an antibody specific for the $\alpha_v\beta_1$ precludes staining for this heterodimer.

**DISCUSSION**

Human $\beta$-cell research is constrained not only by the limited availability of pancreatic tissue, but also by the inherent problem of deriving pure $\beta$-cells that are both viable and sufficient in number. In this study we have adapted conventional adhesion and migration assays to allow an assessment of $\beta$-cell responses using human fetal and adult pancreatic preparations without destructive sorting or enrichment steps. Based on this approach, and other techniques, we now provide the first detailed assessment of the impact of $\alpha_v$-integrin utilization on primary human $\beta$-cell function. Our studies have identified $\alpha_v\beta_5$ and $\alpha_v\beta_1$ as the primary $\alpha_v$-heterodimers utilized by $\beta$-cells for either adhesion or migration on VN. Integrin $\alpha_v\beta_1$ is shown to have an essential and dominant role in promoting fetal $\beta$-cell spreading and motility. In contrast to their fetal counterparts, adult $\beta$-cells fail to either spread or migrate on VN and this deficit may be attributed to a developmental downregulation of $\alpha_v\beta_1$ expression.

The finding that $\alpha_v\beta_1$ has a primary function in supporting fetal $\beta$-cell spreading and migration is consistent with other reports that have linked $\alpha_v\beta_1$ to cell spreading and motility (33-36). In this regard $\alpha_v\beta_1$ has been described as a low affinity receptor that may facilitate the continuous formation and dissolution of adhesion sites, an important requirement for cell motility (36). Interestingly, the developmental decline in $\alpha_v\beta_1$-expression and motility described in this study has also been documented in other cell systems. For example, expression of $\alpha_v\beta_1$ is downregulated following the maturation of embryonic astrocytes and oligodendrocyte progenitors and this loss of expression correlates with a reduced capacity for spreading and migration (33, 34, 37). Accordingly, it is speculated that $\alpha_v\beta_1$ plays an important instructive role in the migration and...
cellular repositioning of early cell lineages during development (33, 34). The subsequent developmental loss of \( \alpha_v \beta_1 \) expression is further proposed to be an important cue for stabilizing static cell-matrix interactions once development is complete (33, 34). Based on these observations and our findings, it is conceivable that \( \alpha_v \beta_1 \) could play a similar instructive role in motile processes required for the formation of early fetal islets. Once islet neogenesis is complete, \( \alpha_v \beta_1 \) expression may be downregulated in order to ensure that inappropriate migration into the surrounding stroma is circumvented and islet integrity is maintained.

We found no evidence that \( \alpha_v \beta_1 \) down-regulation in adult pancreatic cells is attributable to a decrease in the expression of either the \( \alpha_v \)- or \( \beta_1 \)-subunit. This said, an alternative mechanism, perhaps based on the regulation of \( \alpha_v \)- and \( \beta_1 \)-pairing, may be evoked. Recently, it has been proposed that formation of the \( \alpha_v \beta_1 \) heterodimer is strictly regulated by the amount of \( \alpha_v \)-subunit that is available after the preferential formation of other \( \alpha_v \)-containing heterodimers (e.g. \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \)) (36). According to this mechanism, the amount of free \( \alpha_v \)-subunit available for pairing with \( \beta_1 \) in the adult pancreas may be reduced below that in the developing fetal pancreas. Our own studies indicate that \( \alpha_v \beta_5 \) remains highly expressed in the adult pancreas suggesting that most of the available \( \alpha_v \)-subunit is associated with the \( \beta_5 \)-subunit.

Integrin \( \alpha_v \beta_5 \) was found to make a significant contribution to adhesion by both fetal and adult \( \beta \)-cells as well as by fetal and adult PECs. Although blocking \( \alpha_v \beta_5 \) ligation was found to partially inhibit fetal \( \beta \)-cell migration it is unclear whether this is due to abrogation of motility or initial attachment. In this regard, \( \alpha_v \beta_5 \) was not able to support significant fetal \( \beta \)-cell migration when \( \beta_1 \)-integrin ligation was blocked, and adult \( \beta \)-cells were unable to migrate despite using \( \alpha_v \beta_5 \) for adhesion. These observations and the continued expression of \( \alpha_v \beta_5 \) in adult pancreas suggest that \( \alpha_v \beta_5 \) may be more important for maintaining or stabilizing \( \beta \)-cell-matrix interactions. This possibility is supported by other studies that have shown that \( \alpha_v \beta_5 \) expression is maintained or increases with cellular differentiation and upon completion of motile developmental processes (33). However, the capacity of \( \alpha_v \beta_5 \) to support motility may be dependent on cell type since this integrin did support significant PEC migration.
In contrast to both \( \alpha_v\beta_5 \) and \( \alpha_v\beta_1 \), we did not observe a significant role for integrin \( \alpha_v\beta_3 \) in the adhesion or migration of \( \beta \)-cells or PECs and only low levels of expression were detected in fetal or adult pancreatic preparations. It is conceivable, however, that \( \alpha_v\beta_3 \) could assume a more significant role \textit{in vivo} if the extracellular milieu includes growth factors such as hepatocyte growth factor, which are known to increase the expression and/or activity of this integrin.

The participation of \( \alpha_v\beta_5 \) and \( \alpha_v\beta_1 \) in islet development will necessarily be governed by the availability of suitable ligands in the ECM. In this regard, VN has been described in the developing human pancreas both in association with ductal epithelial cells and a subset of insulin-expressing cells (17). Based on its distribution, it has been suggested that localized VN biosynthesis and deposition could serve as an important cue for migratory or morphogenic events required for islet neogenesis (17). In addition to VN, the phosphoprotein osteopontin is also recognized by both \( \alpha_v\beta_5 \) and \( \alpha_v\beta_1 \) (38) and this ECM component has also been described in association with human pancreatic ducts and islets (39).

Given our finding that \( \alpha_v\beta_1 \) can serve as primary mediator of fetal \( \beta \)-cell migration, it is significant that a cyclic-RGD peptide that blocks \( \alpha_v \)-integrin ligation has also been shown to profoundly disrupt the development of islets in an in vivo model (17). Inhibition of integrin-mediated attachment and motility by this peptide is proposed to result in disorganized islet cell clusters that remained closely associated with the ductal epithelium (17). The peptide used in this study contains the motif ARGDN, which interacts with several RGD-dependent integrins including \( \alpha_v\beta_3 \) and \( \alpha_v\beta_1 \) (40, 41). Based on our observations, the marked impact of this peptide on islet neogenesis could, at least in part, be attributed to the inhibition of \( \alpha_v\beta_1 \)-mediated motility.

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REFERENCES

FIGURE LEGENDS

FIG. 1. **Adhesion and spreading of fetal and adult β-cells and PEC on VN.** A, levels of β-cell and PEC attachment to VN over time was assessed. Adherent β-cells were detected by staining for insulin and PEC were visualized by staining with toluidine blue. The percentage of β-cells or PEC attaching after 30-180 minutes was determined as described in the methods section. Treatments were performed in duplicate and results expressed as mean percent adhesion ± S.D. B, β-cell or PEC spreading after 90 minutes on VN was determined by counting round and spread cells per field (20x objective). Adherent β-cells were detected by staining for insulin and PEC were visualized by staining with toluidine blue. Treatments were performed in duplicate and results are expressed as mean percent spreading ± S.D. *Student's T-test analysis on repeat experiments confirmed a significant difference in adult and fetal β-cell spreading at p<0.01.

FIG. 2. **Morphology of fetal and adult β-cells and PEC after attachment to VN.** A and B, Photomicrographs of fetal and adult insulin-positive cells (red) after 90 minutes on VN (40x objective). C and D, Double immunofluorescent staining for insulin (green) and actin (red) in fetal and adult β-cells after 90 minutes on VN (60x objective). E and F, toluidine blue staining of fetal and adult PEC after 90 minutes on VN. Note that PEC have well developed lamellipodia and appear larger than adjacent β-cells (arrowed). Occasional fibroblasts (Fb.) are distinguished by a characteristic elongated morphology (40x objective).

FIG. 3. **β-cells and PEC utilize multiple integrins to attach to VN but αvβ1 is required for β-cell spreading.** A-D, adhesion of fetal and adult β-cells and PECs to VN in the presence or absence of function blocking antibodies to β1 (P4C10), αv (L1A3), αvβ5 (PIF6), or αvβ3 (LM609). Control wells were treated with BSA alone. After 90 minutes the number of adherent β-cells and PEC were determined by staining for insulin (β-cells) followed by toluidine blue (PEC). Adhesion was quantified by counting the number of adherent cells per field (20x objective). Treatments were performed in duplicate and 8 fields were scored per well. Results are expressed as mean adhesion...
as a percent of control (no antibody treatment) ± S.E (n=3). E and F, spreading of fetal β-cells and PEC on VN in the presence or absence of function blocking antibodies. Spreading was assessed after 90 minutes by counting spread versus round cells per field (20x objective). Treatments were performed in duplicate and 8 fields were scored per well. Results are expressed as mean spreading as a percent of control (no antibody treatment) ± S.E (n=3). A-F, *Student's T-test significance at p<0.05 or p<0.01.

FIG. 4. β-cell and PEC migration to VN. Transwell migration assays were modified to allow the detection of migrating β-cells or PEC. A, Photomicrograph of a fetal insulin-positive β-cell that has migrated to the underside of a Transwell insert precoated with VN (60x objective). The 8μM pores, which allow for directed cell migration are clearly visible. B, Photomicrograph showing a cluster of fetal PEC that have migrated to VN (60x objective). Migrant β-cells were detected by staining for insulin, and PEC were identified by staining with toluidine blue. Occasional fibroblasts (Fb.) were distinguished from adjacent epithelial cells based on morphology.

FIG. 5. Migration to VN is limited to fetal β-cells and requires αβ1. A, fetal and adult β-cell migration to VN was assessed after 12 hours. Migrant β-cells were quantified by counting the number of insulin positive cells per insert. Treatments were performed in duplicate and results are expressed as the mean number of migrant cells per insert ± S.D. *Student's T-test of repeat experiments confirmed a significant difference in fetal and adult β-cell migration at p<0.01. B, fetal and adult PEC migration to VN was assessed after 12 hours. Migrant PEC were stained with toluidine blue and counted per field (20x objective). Treatments were performed in duplicate and 8 fields were scored per insert. Results are expressed as mean number of migrant PEC per field ± S.D. C and D, migration of fetal β-cells and PECs to VN in the presence or absence of function blocking antibodies to αv (VNR; 1:25), β1 (P4C10), αvβ3 (LM609), or αvβ5 (PIF6). Results are expressed as mean migration as a percent of control (no antibody treatment) ± S.E (n=3). *Student's T-test significance at p<0.05 or p<0.01.
FIG. 6. Expression of αv-integrins in fetal and adult pancreatic cell preparations. A, αvβ1 expression is downregulated in adult pancreatic cells. Lysates from fetal or adult pancreatic preparations were immunoprecipitated with a pAb to the αv-integrin subunit (AB1930). Immunoprecipitates were then subject to Western blot analysis using antibodies to β1 (LM534), β3 (AP3) and β5 (11D1). B, β1- and αv-integrin subunit expression in total lysates derived from fetal or adult pancreatic cell preparations. Western blot analysis was performed using mAbs LM534 (β1) and LM142 (αv). C, FACS analysis of surface integrin expression associated with fetal and adult pancreatic cell preparations. Single cell preparations were stained with antibodies to human αv (IMM-1044), αvβ3 (PM6/13), αvβ5 (P1F6) or β1 (PC410).
A. Adhesion

% Adhesion

β-cells
PEC
Fetal
Adult

30 90 180 minutes

B. Spreading

% Spread Cells/Field

β-cell
PEC
Fetal adult
Fetal adult

*
Fig. 3
Kaido et al.
Fig. 4
Kaido et al.
Fig. 5
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A. \(\alpha_v\)-immunoprecipitate  

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B. Lysates  

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C. FACS analysis  

**Fetal**  

- Cont.  
- \(\beta_3\)  
- \(\beta_5\)  
- \(\alpha_v\)  
- \(\beta_1\)

**Adult**  

- Cont.  
- \(\beta_3\)  
- \(\beta_5\)  
- \(\alpha_v\)  
- \(\beta_1\)
Alphav-integrin utilization in human β-cell adhesion, spreading and motility
Thomas Kaido, Brandon Perez, Mayra Yebra, Jesse Hill, Vincenzo Cirulli, Alberto Hayek and Anthony Montgomery

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