Molecular Medicine 7(2): 107–114, 2001

Regulation of Laminin 1–Induced Pancreatic β-Cell Differentiation by α_6 Integrin and α-Dystroglycan

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Contributed by L.C. Harrison. Accepted October 2, 2000.

Abstract

Background: The ability to manipulate the development of pancreatic insulin-producing β cells has implications for the treatment of type 1 diabetes. Previously, we found that laminin-1, a basement membrane trimeric glycoprotein, promotes β-cell differentiation. We have investigated the mechanism of this effect, using agents that block the receptors for laminin-1, α_6 integrin, and α-dystroglycan (α-DG).
Materials and Methods: Dissociated cells from 13.5-day postcoitum (dpc) fetal mouse pancreas were cultured for 4 days with laminin-1, with and without monoclonal antibodies and other agents known to block integrins or α-DG. Fetuses fixed in Bouin's solution or fetal pancreas cells fixed in 4% paraformaldehyde were processed for routine histology and for immunohistology to detect hormone expression and bromodeoxyuridine (BrdU) uptake.
Results: Blocking the binding of laminin-1 to α_6 integrin with a monoclonal antibody, GoH3, abolished cell proliferation (BrdU uptake) and doubled the number of β cells. Inhibition of molecules involved in α_6 integrin signaling (phosphotidylinositol 3-kinase, F-actin, or mitogen-activated protein kinase) had a similar effect. Nevertheless, β cells appeared to develop normally in α_6 integrin-deficient fetuses. Blocking the binding of laminin-1 to α-DG with a monoclonal antibody, IIH6, dramatically decreased the number of β cells. Heparin, also known to inhibit laminin-1 binding to α-DG, had a similar effect. In the presence of heparin, the increase in β cells in response to blocking α_6 integrin with GoH3 was abolished.
Conclusions: These findings reveal an interplay between α_6 integrin and α-DG to regulate laminin-1–induced β-cell development. Laminin-1 had a dominant effect via α-DG to promote cell survival and β-cell differentiation, which was modestly inhibited by α_6 signaling.

Introduction

Lineage differentiation of pancreatic β cells has been extensively studied (1–3). However, regulation of β-cell development by extracellular factors remains poorly understood and is a barrier to the cure of type 1 diabetes. Generally, two categories of extracellular factors cooperatively mediate cell growth, differentiation, and survival: soluble hormones and growth factors and cell-associated extracellular matrix (ECM) proteins (4). Previously, we found that laminin-1, a major ECM protein in the basement membrane, promotes differentiation of fetal pancreatic cells into β cells in vitro (5). Two types of receptors for laminin-1, α_6 integrins and α-dystroglycan (α-DG), have been identified in epithelial tissues (6). Integrins are a well-characterized family of heterodimeric cell adhesion molecules composed of noncovalently bound α (120–180 kDa) and β (90–110 kDa) subunits, of which 16 and 8 isoforms, respectively, are known. The α_6 integrin subunit is expressed in a wide range of tissues including the pancreas (7–10), although its expression in fetal mouse pancreas has not been reported. The α_6 subunit dimerizes with the β_1 or β_4 subunit to form $\alpha_6\beta_1$, or $\alpha_6\beta_4$ integrin (6). ECM proteins including laminin-1 bind to integrin receptors to trigger receptor aggregation, actin cytoskeleton polymerization, and activation of tyrosine kinases and signaling cascades leading to growth, differentiation, or apoptosis (11–13). Focal adhesion and actin polymerization following integrin ligation may align signaling molecules in the phosphotidylinositol 3-kinase and the mitogen-activated protein (MAP) kinase pathways to facilitate signal transduction (11,12).

α-DG is a nonintegrin, highly glycosylated peripheral membrane protein identified initially in muscle (14) and subsequently in other tissues, including adult pancreas (15). It is associated with a

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membrane-spanning protein, β-DG, in the dystrophin-glycoprotein complex. In muscle, the complex is structurally organized into three distinct subcomplexes: the dystroglycans (α-DG and β-DG), the sarcoglycans (SGs), and the cytoskeletal proteins dystrophin, syntrophin, and dystrobrevin (16,17). α-DG associates with the F-actin cytoskeleton through dystrophin (18,19). However, SGs are not expressed in epithelial cells (20) and signaling downstream of α-DG has not been characterized. In the present study, we investigated the roles of α_6 integrins and α-DG in laminin-1–induced β-cell development.

Materials and Methods

Mouse Fetal Pancreas Cell Culture

Pancreata were dissected from 13.5 days postcoitum (dpc) fetuses and dissociated into single cells as described (5). Cells were counted in a hemocytometer and viability determined by Trypan blue dye exclusion. Each fetal pancreas yielded approximately 25,000 viable cells (25,694 6 1324; n 5 20). Dissociated cells were plated in eight-chamber slides (Nunc, Naperville,) at 1.5 3 10^4 cells/well in 0.3 ml HYBRIDOMA medium supplemented with 500 UI/ml penicillin and 500 μg/ml streptomycin (GibcoBRL Life Technologies, Gaithersburg, Md) and 200 μg/ml laminin-1 purified from murine Engelbreth-Holm-Swarm tumor basement membrane (GibcoBRL), with or without various concentrations of antibodies and reagents. They were cultured in 10% CO_2 90% air at 378C for 4 days.

Antibodies and Reagents

Rat monoclonal antibody (clone NKI-GoH3, IgG2a) that specifically blocks laminin-1 binding to α_6 integrins (9,21,22) was from Chemicon International (Temecula,). Blocking mouse monoclonal antibodies to integrin α_3 (clone P1B5) (23) and integrin β_4 (clone 3E1) (24) were from GibcoBRL. Blocking mouse monoclonal IgM antibody to α-DG, IIH6, was generously provided as hybridoma supernatant by Dr. Kevin Campbell, Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City. The IgM concentration of IIH6 hybridoma supernatant was estimated from Coomassie staining in SDS-PAGE against serial dilutions of purified mouse IgM standard. Rat monoclonal IgG2a (control for NK1-GoH3), mouse IgM (control for IIH6), and blocking rat monoclonal IgG2a to integrin β_1 (clone 9EG7) (8,9,25) from Pharmingen (San Diego, Calif) were dialyzed against HYBRIDOMA medium at 48C prior to use. Guinea pig antiserum to porcine insulin was from Dako (Glostrup, Denmark). Mouse monoclonal IgG2a to bromodeoxyuridine (BrdU, clone BU-1) was from Amersham Life Science (Buckinghamshire, UK). Rabbit antiserum to porcine glucagon and to human somatostatin and pancreatic polypeptide were from Dako. Fractionated rabbit antiserum to human α-amylase, a marker of acinar cells, and to laminin, were from Sigma Chemicals (St. Louis, Mo).

Heparin, a known blocker of laminin-1 binding to α-DG (18,26), was from Sigma. Wortmannin and Ly294002, inhibitors of PI3K (27,28), genistein and herbimycin, inhibitors of Src family tyrosine kinases (29) associated with focal adhesion kinase (FAK) (30,31), and PD98059, an inhibitor of the MAP kinase kinase, MEK1 (32), were from Calbiochem (La Jolla, Calif). Cytochalasin D, an inhibitor of actin polymerization, was from Sigma.

Immunoperoxidase Staining and Cell Quantitation

Fetuses at 15.5 and 18.5 dpc from homozygous (2/2) or heterozygous (1/2) α_6 integrin gene targeted (33) or wild-type mice were fixed overnight in Bouin's solution. After standard dehydration processing, fetuses were embedded into paraffin and sectioned at 7 μm. Cultured pancreatic cells were washed three times with warm mouse tonicity phosphate-buffered saline (MT-PBS) and fixed with 4% paraformaldehyde (PFA) for 10 min. Endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 8 min. Prior to antibody staining, nonspecific protein binding was blocked by incubation for at least 30 min with MT-PBS containing 2% bovine serum albumin or 2% normal rabbit serum. Controls were performed by replacing first antibody with preimmune serum from the appropriate species. Cells were incubated with primary antibodies for 90 min at room temperature, followed by three thorough washes with MT-PBS. Horseradish peroxidase-conjugated rabbit anti-guinea pig or swine anti-rabbit immunoglobulins (Dako) were added for 30 min at room temperature followed by thorough washes. Immunoperoxidase was detected with 3,39-diaminobenzidine/H_2O_2 for 4–8 min, and slides counterstained with hematoxylin.

Immunoperoxidase-positive and -negative cells were counted in the central strip of each culture chamber (90 3 90 mm square) under a microscope equipped with an eyepiece graticule (Olympus, Japan) at 3 40 power and calibrated with a micrometer (Olympus).

Immunofluorescence Staining

In some cultures, 100 μM BrdU (Sigma) was included to label proliferating cells; cells were fixed with 4% PFA at days 1, 2, 3, and 4 for insulin and BrdU double immunofluorescence staining. Pretreatment and primary antibody incubations were as described above, followed by incubation with Texas Red-conjugated goat anti-guinea pig immunoglobulins (Vector Laboratories, Burlingame,) or fluorescein isothiocyanate–conjugated rabbit anti-mouse, -rat, or -goat or swine anti-rabbit immunoglobulins (Dako) for 30 min at room temperature and three thorough washes. Slides were observed and photomicrographed under a Zeis Axiophot fluorescence microscope.

Statistics

Dose responses were analyzed by ANOVA and differences between groups were analyzed by the nonparametric Mann-Whitney U test. Data are presented as mean ± SEM of at least three independent experiments.

Results

Laminin-1 was detected by immunofluorescence on dissociated fetal mouse pancreas cells only after its addition to culture medium (data not shown), indicating that these cells produce little if any laminin-1 in culture. Although both pancreatic epithelial cells and vimentin-positive mesenchymal cells (60% of total initially) attached, the latter did not survive in low-cell density, serum-free conditions [0% vimentin-positive after 4-day culture, see also (5)]. The epithelial cells expressed α_6 integrins and α-DG to which the added laminin bound [data not shown; see also (6)].

α_6 Integrin Blockade Stimulates β-Cell Differentiation

To investigate the role of α_6 integrin in laminin-1–induced β-cell differentiation, pancreas cells were cultured with laminin-1 and the rat monoclonal antibody GoH3, which specifically blocks laminin-1 binding to α_6 integrins. In the presence of GoH3, cells appeared more uniformly spherical rather than flattened with laminin-1 alone (Fig. 1A). GoH3 induced an increase in the number of both total cells and β cells (Fig. 1B). The increase in total cell number was due to the increase in β-cell number. For example, at 40 μg/ml GoH3, β-cell number per well increased from 2265 ± 240 (mean ± SEM, $n = 3$) to 6886 ± 364 and total cell number from 4016 ± 650 to 7188 ± 228 ($p < 0.05$). Over the dose response, there was a significant increase ($p < 0.01$) from 61–96% in the ratio of β cells to total cells. Rat IgG2a control antibody had no effect on cell number (Fig. 1B). β-Cell number in the presence of GoH3 without laminin-1 was similar to that in medium only (Fig. 1C). Thus, the effect of GoH3 to increase β-cell differentiation depended on the presence of laminin-1, and GoH3 did not mimic the effect of laminin-1. The proportion of glucagon-positive α cells was unchanged in the presence of GoH3 and laminin-1 (data not shown), and somatostatin-positive δ cells, pancreatic polypeptide-positive (PP) cells and amylase-positive acinar cells were not detected.

To measure cell division, 100 μM BrdU was added with laminin-1 (200 μg/ml), with and without GoH3, and BrdU-positive cells analyzed at days 1–4. In the absence of GoH3, 2.5% of cells were BrdU positive; in the presence of GoH3, BrdU-positive cells were not detected.

The α_3 integrin subunit is the only α subunit with significant (40%) identity to the α_6 subunit (34), but the blocking anti-α_3 monoclonal antibody P1B5 (10–40 μg/ml) had no effect on total or β-cell number (data not shown). The α_6 integrin subunit dimerizes noncovalently with either β_1 or β_4 subunits to form $\alpha_6\beta_1$ or $\alpha_6\beta_4$ integrin (6), but neither anti-β_1 (9EG7) nor anti-β_4 (3E1) monoclonal antibodies influenced total or β-cell number (data not shown).

Inhibition of Molecules Involved in α_6 Integrin Signaling Pathways Stimulates β-Cell Differentiation

Specific inhibitors were used to block molecules involved in α_6 integrin downstream signaling. To determine if PI3K has a role in laminin-1–induced β-cell development, 13.5-dpc fetal mouse pancreas cells were cultured for 4 days with the PI3K inhibitors, wortmannin, or Ly294002, at concentrations (0.1-100 μM) reported to be nontoxic for pancreas cells (35). Both wortmannin and Ly294002 significantly increased ($p < 0.05$) total and β-cell numbers in a dose-dependent manner (Fig. 2AB). Without laminin-1, the number of β cells was not affected by either agent, indicating that differentiation in the presence of laminin-1 requires inhibition of PI3K. Blocking formation of F-actin by cytochalasin D may inhibit the Ras-PI3K-MEK1 signaling cascade (13). Cytochalasin D increased laminin-1–induced β-cell differentiation, similar to wortmannin or Ly294002 (Fig, 2C), consistent with a role for the actin cytoskeleton in β-cell differentiation. Inhibition of MEK1, downstream from PI3K, with PD98059 also significantly increased ($p < 0.05$) laminin-1–induced β-cell differentiation (Fig. 2D). Src family tyrosine kinases are associated with FAK, which may signal MAP kinase via Ras (30). However, the Src kinase inhibitors, genistein and herbimycin A, did not affect laminin-1–induced β-cell differentiation (Fig. 2EF).

Islet Cell Development Appears Normal in α_6 Integrin-Deficient Mice

In α_6 integrin-deficient mouse fetuses, the morphology of the pancreas at 15.5 and 18.5 dpc appeared normal. Immunostaining for insulin, glucagon, somatostatin, and pancreatic polypeptide revealed that the distribution and appearance of islet cells were similar in homozygous (2/2) and heterozygous (1/2) mutants and wild-type (1/1) fetuses at 18.5 dpc. At 15.5 dpc, the appearance of α and β cells was similar among 2/2, 2/1, and 1/1 fetuses, and δ and PP cells were not observed (Fig. 3, insulin staining for β cells).

α-Dystroglycan Blockade Inhibits β-Cell Development

To investigate the role of α-DG in laminin-1–induced β-cell development, 13.5-dpc fetal mouse pancreas cells were cultured with laminin-1 and either the mouse IgM monoclonal antibody, IIH6, which blocks laminin-1 binding to α-DG (18,36,37), or

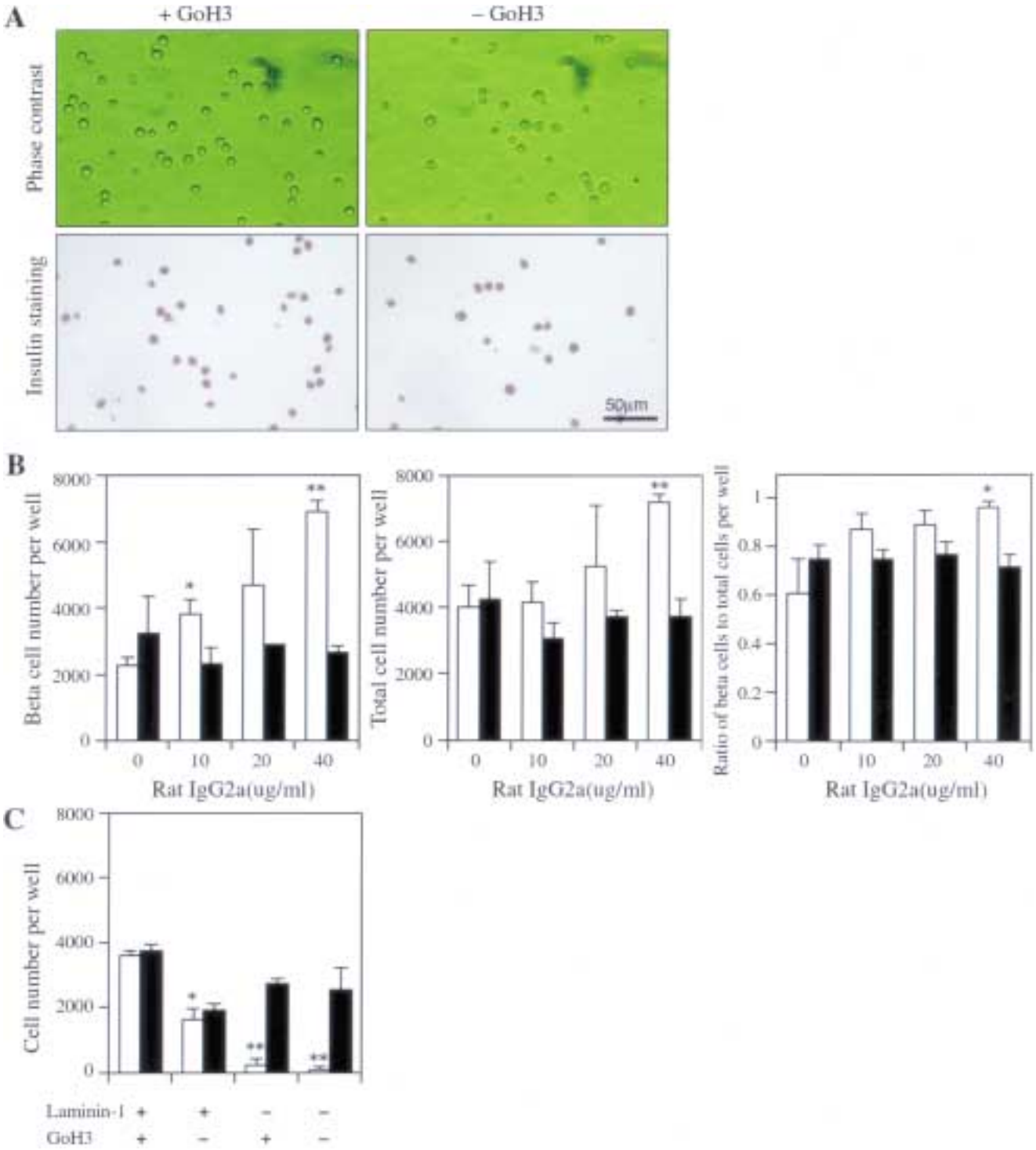

Fig. 1. β-cell differentiation in the presence of laminin-1 (200 μg/ml) and rat GoH3 blocking monoclonal antibody to α_6 integrin. (A) Fetal mouse pancreas cells (13.5 dpc) cultured for 4 days with (1) and without (2) GoH3 (40 μg/ml), under phase contrast microscopy (top panel) and after staining with guinea pig anti-insulin serum and peroxidase (lower panel). (B) Numbers of β cells and total cells after culture of 13.5-dpc fetal mouse pancreas cells for 4 days with GoH3 (open bar) or rat IgG2a isotype control antibody (solid bar). *p , 0.05, **p , 0.01 versus in the absence of GoH3 or in the presence of isotope control antibody. (C) Number of β cellsˆ (open bar) and total cells (solid bar) after culture of 13.5-dpc fetal mouse pancreas cells for 4 days with GoH3 (40 μg/ml) in the presence or absence of laminin-1. *p , 0.001 versus in the presence of both laminin-1 and GoH3. **p , 0.01 versus in the presence of laminin-1 alone. Cell counts, as described in "Materials and Methods," were expressed as mean 6 SEM (n 5 3 separate experiments).

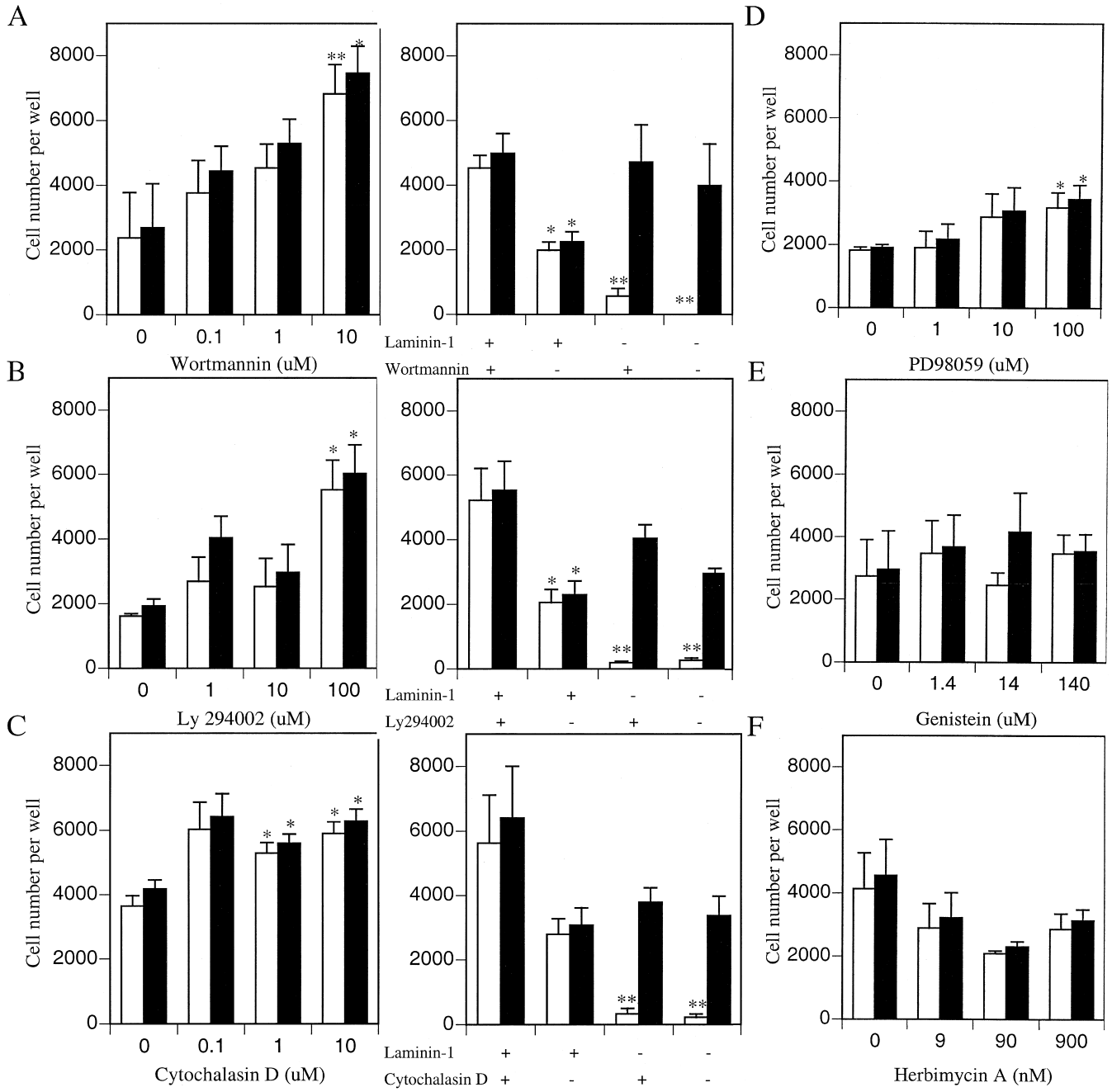

Fig. 2. Number of β cells (open bar) and total cells (solid bar) in the presence of PI3K inhibitor wortmannin (A) or Ly294002 (B), F-actin polymerization inhibitor cytochalasin D (C), MEK1 inhibitor PD98059 (D), and Src family tyrosine kinase inhibitors genistein (E) and herbimycin A (F). β Cells were identified by staining with guinea pig anti-insulin serum and peroxidase and cell counts were expressed as mean 6 SEM (n 5 3 separate experiments). The left panels show number of β cells and total cells in the presence of laminin-1 (200 μg/ml) and the indicated concentrations of inhibitors. *p , 0.05, **p , 0.01 versus in the absence of wortmannin, Ly 294002, cytochalasin D or PD 98059. The right panels show number of β cells and total cells in the presence or absence of laminin-1 (200 μg/ml) and wortmannin (10 μM), Ly294002 (100 μM), or cytochalasin D (10 μM). *p , 0.001 versus in the presence of laminin-1 and wortmannin or Ly294002. **p , 0.01 versus in the presence of laminin-1 alone.

heparin, which also blocks laminin-1 binding to α-DG (17,25). IIH6 had a dose-response effect to significantly decrease (p , 0.01) the number of both total and β cells (Fig. 4A), whereas control mouse IgM at the same concentrations had no effect (data not shown). Heparin at 100 μM also significantly decreased (p , 0.05) the number of total and β cells (Fig. 4B). Furthermore, at this concentration, heparin blocked the effect of the α_6 integrin antibody, GoH3, to increase β-cell differentiation (Fig. 4C).

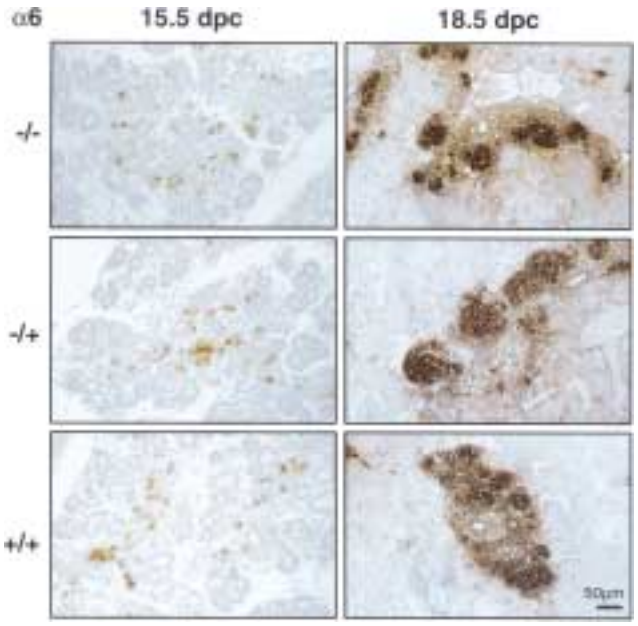

Fig. 3. Pancreas morphology in α_6 integrin gene knockout fetuses at 15.5 and 18.5 dpc. β cells were identified with guinea pig anti-insulin serum and peroxidase in α_6-deficient homozygous (2/2) and heterozygous (2/1) fetuses and wild-type (1/1) control fetuses.

These findings imply that laminin-1 signaling via α-DG promotes cell survival and β-cell differentiation, but that this effect can be inhibited by laminin-1 signaling through α_6 integrin.

Discussion

The number of β cells in fetal mouse pancreas cell cultures was significantly increased, concomitant with abolition of cell division, by blocking laminin-1 binding to α_6 integrin receptors or by inhibiting the PI3K-MAP kinase pathway or actin polymerization downstream of α_6 integrins. These findings indicate that the α_6 integrins mediate a proliferative signal from laminin-1 through the MAP kinase ERK pathway and a net inhibitory effect on β-cell differentiation. Blocking either the β_1 or β_4 integrin subunit had no effect on β-cell differentiation, indicating that the α subunit has a role distinct from its β subunits (38). On the other hand, blocking laminin-1 binding to α-DG with the monoclonal antibody, IIH6, dramatically decreased β-cell number and, furthermore, abrogated the effect of GoH3. Thus, on the basis of these findings we propose that laminin-1 exerts counter-regulatory effects through α_6 integrin and α-DG receptors. The predominant effect, signaling for cell survival and β-cell differentiation through α-DG, is countered in part by signaling through α_6 integrins. This model is depicted schematically in Fig. 5.

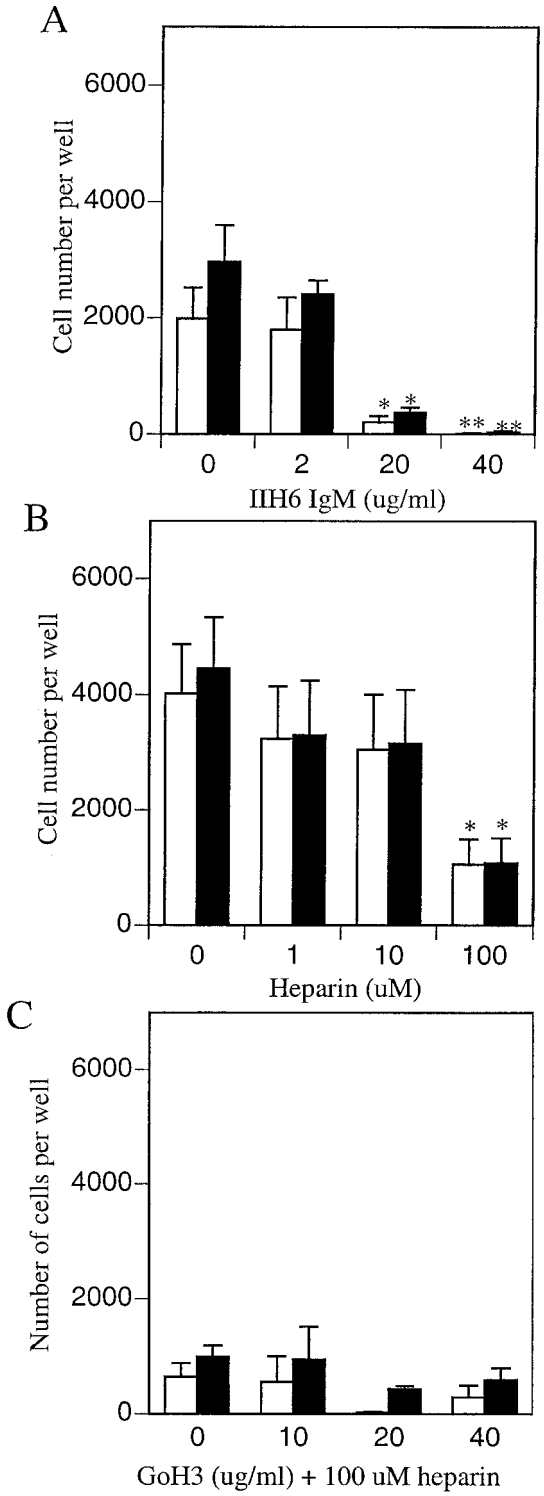

Fig. 4. Number of β cells (open bar) and total cells (solid bar) after culture of 13.5-dpc fetal mouse pancreas cells for 4 days in presence of laminin-1 (200 μg/ml) and IIH6 blocking monoclonal IgM antibody to α-DG (A) or heparin (B), or in presence of laminin-1 (200 μg/ml) and heparin (100 μM) and indicated concentrations of GoH3 rat IgG2a monoclonal antibody to α_6 integrin (C). β Cells were identified with guinea pig anti-insulin serum and peroxidase and cell counts were expressed at mean 6 SEM (n 5 3 separate experiments). *p , 0.005, **p , 0.01 versus in the presence of laminin-1 and IIH6 (A) or heparin (B).

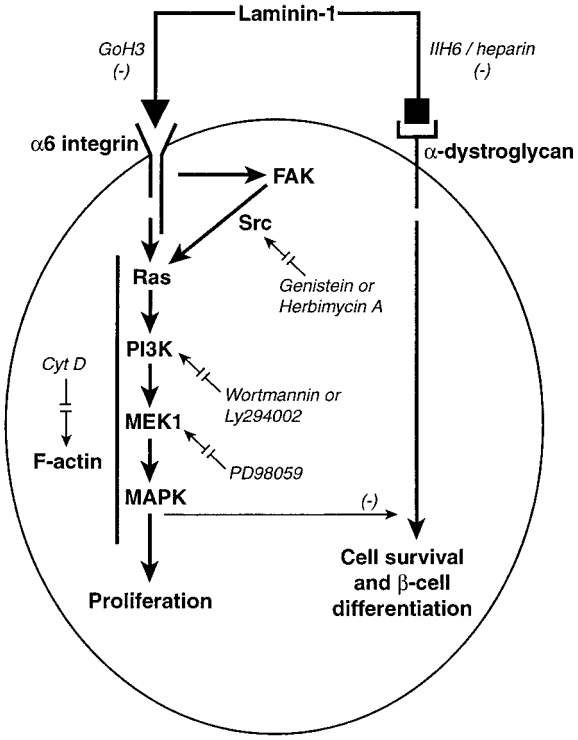

Fig. 5. Dual regulation by laminin 1 to inhibit (via α_6 integrins) and stimulate (via α-DG) β-cell differentiation. Blocking laminin-1 binding to α_6 integrins by GoH3 antibody, or inhibiting PI3K, MEK1 or actin polymerization by wortmannin or Ly294002, PD98059 or cytochalasin D, respectively, significantly increases the number of total and β cells. In contrast, blocking laminin-1 binding to α-DG by IIH6 antibody or heparin decreases significantly the number of total and β cells. In the presence of heparin, the effect of GoH3 to enhance β-cell differentiation is abolished.

The increase in laminin-1–induced β-cell differentiation after inhibition of PI3K with wortmannin or Ly294002 confirms a previous report by Ptasznik et al (39) that this enzyme is a negative regulator of β-cell differentiation. These investigators found that wortmannin induces morphologic and functional endocrine differentiation in human fetal undifferentiated pancreas cells. PI3K is upstream of the MEK1 signal cascade (13). Inhibition of MEK1 with PD98059 also enhanced laminin-1–induced β-cell differentiation. This is consistent with reports that MEK1 is activated by ligation of laminin-1 and $\alpha_{6A}\beta_1$ (40) or $\alpha_6\beta_4$ (24) integrins. Inhibition of Src tyrosine kinases had no effect and therefore this family of kinases is probably not involved in laminin-1–induced β-cell differentiation. In summary, these data are consistent with the view that α_6 integrin signaling through the MAP kinase ERK module exerts a negative regulatory effect on β-cell differentiation.

On the basis of these findings in vitro, the absence of α_6 integrins in vivo might be expected to promote β-cell development. However, this did not appear to be the case. There are a number of possible explanations why β-cell development may be normal in α_6 integrin-deficient mice. The first, in agreement with the present findings, is that inhibition through α_6 integrin is relatively modest in comparison with the survival-differentiation signal through α-DG. Second, α_6 integrin deficiency could be compensated for by other integrin members (41), although we found that blocking the related α_3 integrin had no effect on laminin-1–induced β-cell development. Third, laminin-1 signaling through α_6 integrins could be antagonized in vivo by signals from other ECM proteins (e.g., collagen IV) (5). Overall, compared with α-DG, α_6 integrins may play a contributory but not essential role in β-cell differentiation. A key role for α-DG transducing survival/differentiation signals from laminin-1 is supported by a recent report from Montanaro et al (42) in which transfection of myoblasts with antisense α-DG RNA was shown to decrease the number of myotubes in culture. Our findings indicate that laminin-1 has dual effects on β-cell development via α_6 integrins and α-DG, but that its binding to α-DG dominates in promoting survival and β-cell differentiation. Understanding mechanisms by which extracellular factors promote β-cell development should facilitate β-cell replacement therapy for type 1 diabetes.

Acknowledgments

This study was supported by the Juvenile Diabetes Foundation International (Angelo and Susan Alberti Program Grant) and by the National Health and Medical Research Council of Australia. The authors thank Dr. Jorge Gonez for helpful advice and Margaret Thompson for secretarial assistance.

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