Laminin-1 Promotes Differentiation of Fetal Mouse Pancreatic β-Cells

Fang-Xu Jiang, David S. Cram, Henry J. DeAizpurua, and Leonard C. Harrison

Extracellular factors that regulate the growth and differentiation of cell lineages in the pancreatic primordia are poorly understood. Identification of these factors for pancreatic islet β-cells could open new avenues for the treatment of insulin-dependent diabetes. We developed a low cell density serum-free culture system for dissociated pancreatic cells from the 13.5-day mouse fetus and investigated the effects of extracellular matrix proteins on differentiation of islet cells. After 4 days in culture, total cell number decreased by two-thirds, but insulin-positive β-cell number increased 10-fold. Both of collagens I and IV inhibited cell survival (by >50%), whereas fibronectin had no effect. In the presence of soluble laminin-1, however, the number of β-cells increased linearly by 60-fold without an increase in the total cell number; glucagon-positive cell number was unchanged, and somatostatin and pancreatic polypeptide-positive cells were not detected. The effect of laminin-1 was completely blocked by a monoclonal rat anti–laminin-1 antibody. In the presence of laminin-1, the thymidine analogue, BrdU, was incorporated into only 2.5% of cells, which were mainly insulin-negative at days 1–3. Laminin-1 appeared, therefore, to induce differentiation of β-cells from precursor cells in day-13.5 fetal pancreas. Laminin-1 was shown to be expressed in the epithelial basement membrane of the 13.5- to 17.5-day fetal pancreas. These findings provide the first evidence of a role for laminin-1 to promote differentiation of pancreatic β-cells. *Diabetes* 48:722–730, 1999

The mammalian pancreatic primordia evaginate from foregut endoderm in early fetal life (1) and differentiate into two distinct tissue types: endocrine tissue, the islets of Langerhans, which secretes hormones into the bloodstream, and exocrine tissue, which secretes digestive enzymes into the intestinal tract. The islets contain four main types of endocrine cells, which synthesize insulin, glucagon, somatostatin, and pancreatic polypeptide. The ontogeny of islet cells has been studied extensively (2). All four types of endocrine cell are believed to arise from common multipotent precursors that express the PDX-1 (also called IPF-1, STF-1, and IDX-1) transcription factor (3) and coexpress several hormones and neuronal markers as they begin to differentiate (4). Cells coexpressing glucagon and insulin first become evident in the pancreatic primordia at 9.5 days postcoitum (dpc) in mice, but their number decreases with further development. Somatostatin-secreting cells first appear at 14.5 dpc, and pancreatic polypeptide-producing cells appear around birth (4,5). PDX-1 is required for the outgrowth of pancreatic primordia (6,7). Gene knockout studies reveal important roles for several transcription factors in islet cell development. ISL-1 is required for the formation of dorsal mesenchyme, and thereafter for the generation of dorsal islet cells (8), Pax4 and Pax6 for the differentiation of β-cells (9) and α-cells (10), respectively, and BETA2/NeuroD for the development of both endocrine and exocrine cells (11).

The factors and mechanisms that regulate lineage differentiation of islet cells from multipotent precursors are poorly understood. Their identification could lead to new approaches to the treatment of β-cell destruction in insulin-dependent diabetes. Broadly speaking, two classes of cooperative signals guide cellular proliferation, differentiation, and apoptosis: soluble signals including hormones and growth factors and cell-associated signals delivered by extracellular matrix (ECM) proteins (12). The ECM that contacts directly with epithelial cells is the basement membrane (BM). All BMs are composed of two relatively distinct protein layers of collagen and laminin polymers (13), linked by specific interactions with nidogen/entactin (14). The role of ECM proteins in the growth and differentiation of the pancreas has attracted increasing attention in recent years. Fetal pancreatic epithelia grown without mesenchyme in a commercially available BM gel, Matrigel, formed cystic epithelial structures, with some epithelial cells differentiating into end-ocrine cells (15). Similarly, when cultured in a three-dimensional collagen I gel, rat pancreatic rudiments without mesenchyme actively differentiated into islet tissue (16). Pancreatic ductal epithelial cells from adult rats grew best on Matrigel, less well on collagen matrix, and very poorly on plastic alone (17). Human pancreatic islets have been transdifferentiated into ductal epithelia in collagen I matrix culture (18,19).

Based on these observations, we hypothesized that ECM proteins may induce the differentiation of fetal pancreatic cells into specific islet cell lineages. To test this hypothesis, we analyzed dissociated pancreatic cells from 13.5-dpc fetal mice cultured under serum-free conditions in the presence of ECM proteins. Our results indicate that laminin-1 specifically promotes differentiation of β-cells from precursor cells at 13.5 dpc.

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BM, basement membrane; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; dpc, days postcoitum; ECM, extracellular matrix; GAF, Gomori aldehyde fuchsin; MT, mouse tonicity; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PP, pancreatic polypeptide; RT, reverse transcriptase.

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RESEARCH DESIGN AND METHODS

Fetal pancreatic cell preparation. Dissociated cells were prepared from 13.5-dpc fetal pancreas of CBA mice housed and bred at the Walter and Eliza Hall Institute of Medical Research. The morning of observing a vaginal plug was designated day 0.5 of gestation. Pregnant mice at 13.5 dpc were killed by cervical dislocation, and fetuses were collected into ice-cold sterile mouse tonicity (MT) phosphate-buffered saline (PBS). Fetal pancreases were harvested under an Olympus dissection microscope into RPMI 1640 medium and dissociated in a shaking water bath at 37°C for 11 min in Ca^{2+}/Mg^{2+}-free PBS containing 0.05% trypsin and 0.02% EDTA, as described for testis by Bucci et al. (20). After dissociation, trypsin was inactivated by addition of 8% bovine serum albumin (BSA) (fraction V; Sigma, St. Louis, MO). The tissue was aspirated several times in a pipette, passed through a 27-gauge needle to make a single cell suspension, and then rinsed in 10 µg/ml DNAse I (Promega, Madison, WI) in RPMI 1640 for 10 min to reduce cellular aggregates. Cell suspensions were filtered through a 70- to 100-µm steel mesh and centrifuged at 400*g* for 5 min, and the supernatant was discarded. The cell pellet was resuspended in Hybridoma medium (GibcoBRL Life Technology, Gaithersburg, MD) and stored on ice. Cells were counted under a hemocytometer, and viability was determined by exclusion of 0.2% trypan blue dye.

Pancreatic cell culture. Pancreatic cells were plated in eight-chamber slides (Nunc, Naperville, IL) at 1.5×10^4 cells/well in 0.3 ml Hybridoma medium supplemented with 500 U/ml penicillin and 500 µg/ml streptomycin. The cells were cultured in 10% CO_2, 90% air at 37°C in 100% humidity for 4 days. This culture period was determined by time-response experiments (see Fig. 5*A*).

ECM proteins. Four major ECM proteins were examined. Collagen I was prepared from rat tail tendons as described by Lee et al. (21) and sterilized by filtration through a 0.2-µm filter. Both collagen I and murine collagen IV (GibcoBRL) were diluted in RPMI 1640 medium and added to eight-chamber slides (Nunc) at 37°C for 3–4 h. The slides were washed three times with RPMI medium before plating cells. Laminin-1, prepared from murine Engelbreth-Holm-Swarm (EHS) tumor BM

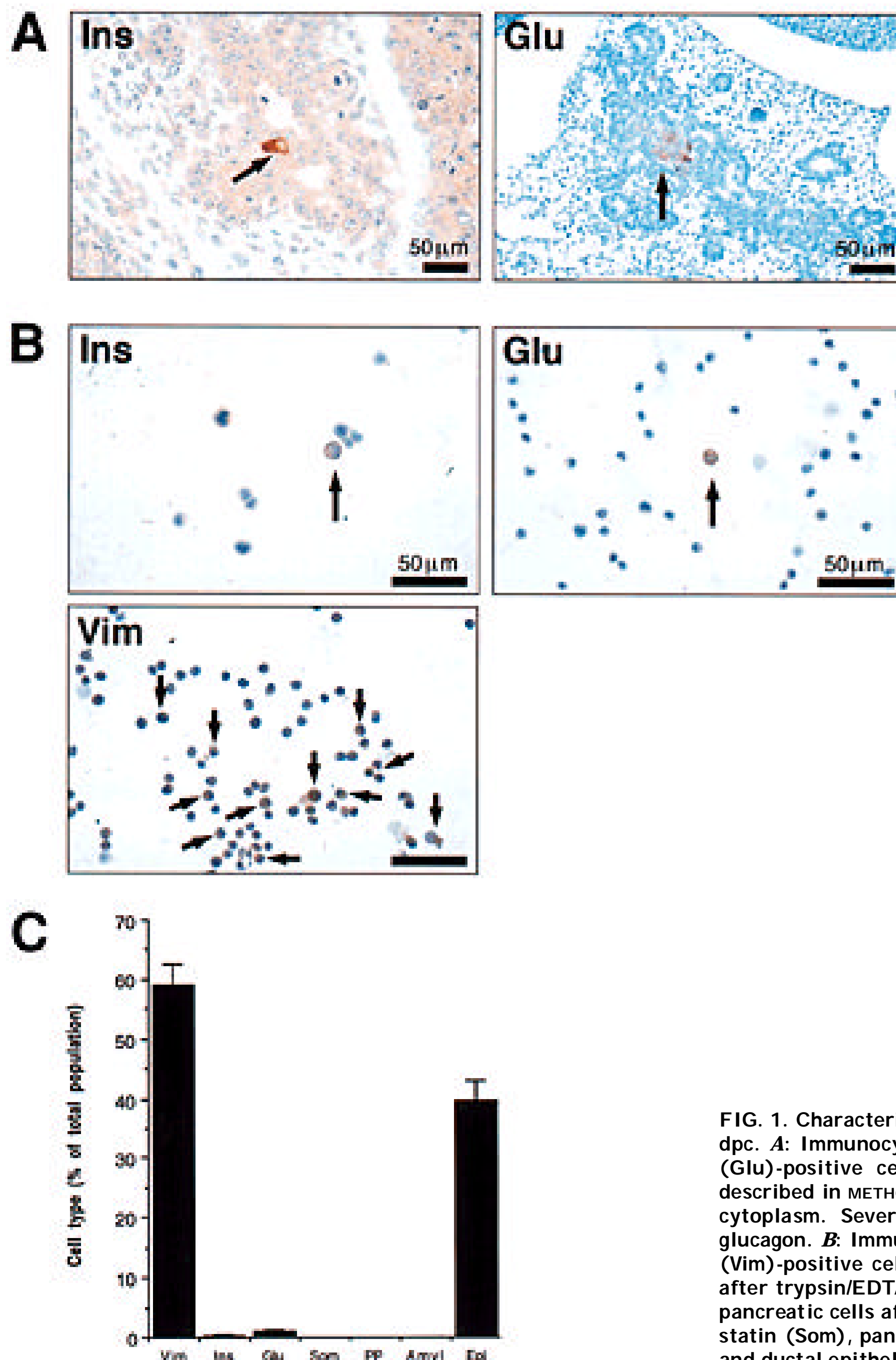

FIG. 1. Characterization of fetal mouse pancreatic cells at 13.5 dpc. *A*: Immunocytochemistry for insulin (Ins)- and glucagon (Glu)-positive cells in the fetal mouse pancreas tissue as described in METHODS. Note β-cell ( ) expressing insulin in the cytoplasm. Several α-cells ( ) express various amount of glucagon. *B*: Immunocytochemistry for Ins-, Glu-, and vimentin (Vim)-positive cells ( ) in cytospins of fetal mouse pancreas after trypsin/EDTA dissociation. *C*: Quantitation of dissociated pancreatic cells after immunostaining for Ins, Glu, Vim, somatostatin (Som), pancreatic polypeptide (PP), and amylase (Amyl) and ductal epithelial cells (Epi) of cytospins.

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(22), was purchased from GibcoBRL. To prevent polymerization, laminin-1 was maintained at 4°C and used directly or first dialyzed at 4°C against Hybridoma medium. Laminin-1 and murine fibronectin (GibcoBRL) were diluted directly into the chilled medium and then added to the cells.

Antibodies. Guinea pig anti-porcine insulin antiserum (1:200) (Chemicon, Temecula, CA, or Dako, Glostrup, Denmark) and mouse monoclonal anti-insulin IgG1 (clone Z006) (1:100) were purchased from Zymed (San Francisco, CA). Mouse monoclonal anti-BrdU IgG2a (Clone BU-1) was obtained from Amersham Life Science (Buckinghamshire, U.K.). Mouse monoclonal anti-vimentin IgG1 (clone V9) (1:20), a marker of mesenchyme-derived cells, rabbit antisera to porcine glucagon (1:100), human somatostatin (1:200), and pancreatic polypeptide (1:500) were purchased from Dako. Fractionated rabbit antiserum to human α-amylase, a marker of acinar cells, was from Sigma. Rat monoclonal anti-mouse laminin-α1 (clone 198), -α2 (clone 4H8), and -α5 (clone 4G6) antibodies were kindly provided by Dr. L. M. Sorokin (Institute of Experimental Medicine, University of Erlangen-Nurnburg, Germany) (23). Rat monoclonal anti–laminin-1 antibody IgG2b (clone LAM-1; ICN Biochemicals, Aurora, OH) and rat IgG2b control antibody (Pharmingen, San Diego, CA) were dialyzed against Hybridoma medium at 4°C before use.

Immunocytochemistry. Fetuses from 13.5- and 17.5-dpc mice were fixed overnight with 4% paraformaldehyde in MT-PBS at room temperature. After standard dehydration processing, fetuses were embedded into paraffin and sectioned at 7 µm. The sections were dewaxed, rehydrated, and washed with MT-PBS. For cryostat sections, fetuses were directly snap-frozen in liquid nitrogen and sectioned at 8 µm. The sections were air-dried for 40–60 min and fixed in cold (–2°C) acetone for 10 min for immunofluorescence staining. Cytospins of dissociated cells were air-dried for 40 min and fixed with 4% paraformaldehyde for 10 min. After 4 days of culture, cells were washed twice with warm MT-PBS and fixed with 4% paraformaldehyde for 10 min. In some experiments, 100 µmol/l bromodeoxyuridine (BrdU) (Sigma) was added (24) with laminin-1 to label proliferating cells, and cells were fixed at days 1, 2, 3, and 4 for insulin and BrdU double immunofluorescence staining.

For immunoperoxidase staining, endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 8 min. Before antibody staining, non–specific protein binding was blocked by incubation for at least 30 min with MT-PBS containing 2% BSA or 2% normal rabbit serum. Controls were performed by replacing first antibody with preimmune serum from the appropriate species. Tissue sections or cells were incubated with primary antibodies for 90 min at room temperature, followed by three thorough washes with MT-PBS. Horseradish peroxidase conjugated secondary rabbit anti–guinea pig, swine anti-rabbit, or rabbit anti-mouse immunoglobulins (1:80; Dako) were added for 30 min at room temperature, followed by thorough washes. Immunoperoxidase was detected with 3,3′-diaminobenzidine/H_2O_2 for 4–8 min, and slides were counterstained with hematoxylin. The specificity for insulin staining in cultured cells was confirmed by preincubating guinea pig anti-insulin serum with 10^{-5} mol/l recombinant human insulin Humulin (Eli Lilly, Indianapolis, IN). On chamber slides, immunoperoxidase-positive and -negative cells were quantitated in the central strip of each well (a 90 × 90 mm square) under ×40 power with an eyepiece graticule (Olympus, Tokyo) calibrated with a micrometer (Olympus).

For immunofluorescence staining, Texas Red conjugated goat anti–guinea pig immunoglobulins (Vector Laboratories, Burlingame, CA) or fluoresein isothyocyanate-conjugated rabbit anti-rat and mouse immunoglobulins (Dako) were incubated for 30 min at room temperature, followed by three thorough washes.

Slides were observed and photomicrographed under a Zeiss Axiophot fluorescent microscope or a Fluovert confocal microscope.

Histocytochemistry. The Gomori aldehyde fuchsin (GAF) stain (25) was used to detect insulin in granules.

Reverse transcriptase–polymerase chain reaction. Reverse transcriptase (RT)-polymerase chain reaction (PCR) was used to detect insulin II mRNA expression. Total RNA was extracted from cultured cells with phenol/guanidine isothiocyanate-based RNAzol B (Cinna/Biotex, Houston, TX) and digested with RNase-free DNase I (Promega) to remove contaminating genomic DNA. RNA was reverse transcribed with mouse mammary tumor virus reverse transcriptase (Promega) in 1× transcription buffer containing 0.5 µmol/l random hexanucleotides (Bresatec, Adelaide, South Australia, Australia) and 400 µmol/l dNTPs. Aliquots of the cDNAs were amplified by PCR in 1× PCR buffer (Perkin Elmer, Branchburg, NJ) containing 200 µmol/l dNTPs, 1 µmol/l of each primer pair, and 1 U Taq polymerase. The primers for insulin (5′ TCTCTACCTGGTGTGTGG 3′, 5′ TTGCAGTAGTTCTCCAGC 3′) and the control housekeeping gene β-actin (5′ GTGGGCCGCCCTAGGCACCA 3′, 5′ CTCTTTGATGTCACGCACGATTTC 3′) yielded PCR products of 217 and 530 bp, respectively. PCR reactions were performed for 35 cycles (95°C, 30 s; 65°C, 30 s; 72°C, 30 s), and amplified products were separated in 1.5% agarose gels.

Statistics. Data derived from three or more independent experiments are reported as means ± SE, and differences were analyzed by Student's *t* test.

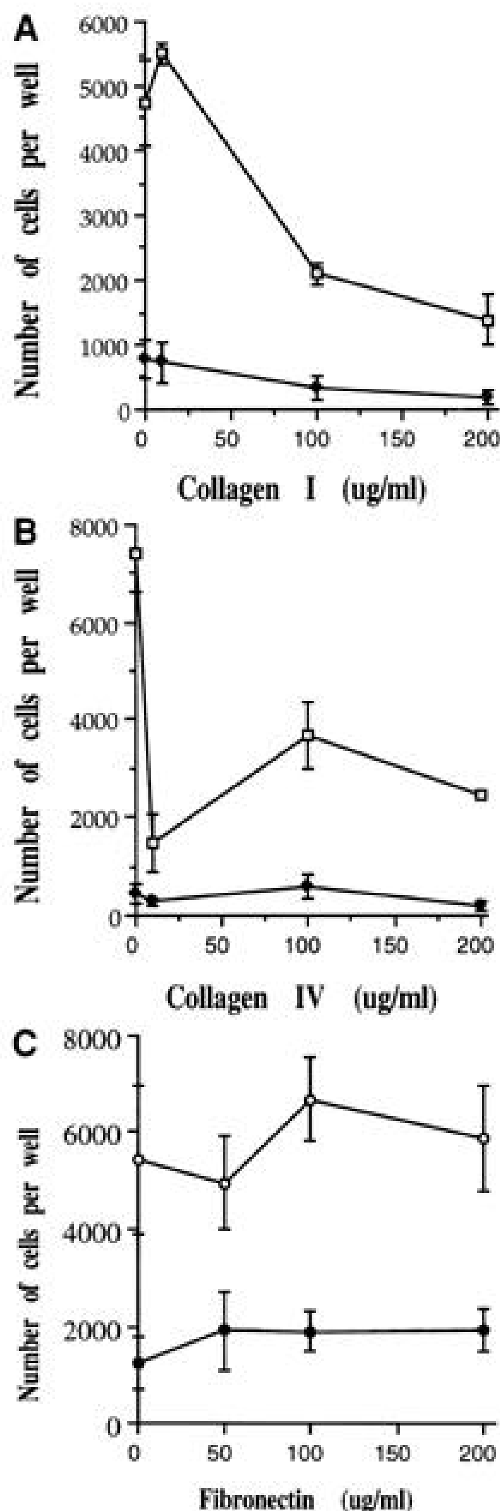

FIG. 2. Effect of collagens I (*A*) and IV (*B*) on total (□) and insulin-positive (●) cell numbers, and fibronectin with (●) or without (○) collagen I (200 µg/ml) (*C*) on total cell number, of 13.5-dpc mouse pancreatic cells (1.5 × 10^4 cells/well plated) in culture for 4 days.

RESULTS

Fetal pancreatic cell characterization. As a baseline, we characterized the 13.5-dpc fetal pancreas at the time of harvest and after cell dissociation. Two types of endocrine cells

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were detected, but amylase-positive exocine cells were not detected. Insulin-positive (β) cells were observed occasionally, whereas glucagon-positive (α) cells, both clustered and scattered in the mesenchymal tissue, were predominant (Fig. 1*A*). Somatostatin (δ)- and pancreatic polypeptide (PP)-positive cells were not detected at 13.5 dpc, but first appeared at 15.5 and 17.5 dpc (not shown), respectively. The timed appearance of these endocrine cells is consistent with previous studies (2,4,5,26). After dissection and dissociation with trypsin/EDTA, the 13.5-dpc fetal pancreas yielded ~2 $\times$ 10^4 cells (20797 ± 1849, n = 19) that were >95% viable as determined by trypan blue dye exclusion. These dissociated cells were cytospun onto slides and analyzed immunocytochemically (Fig. 1*B*). The major proportion (59%) of the cells exhibited staining for the mesenchymal marker vimentin (Fig. 1*C*), consistent with the quantitation based on cellular morphology 2 h after plating (see below). α-Cells constituted 1% of the total population, whereas β-cells were only 0.2% of the total population (Fig. 1*C*). Again, δ- and PP-cells were not observed. The remaining 30% of the population was uncharacterized, but presumably comprised mainly epithelial cells (Fig. 1*C*). We attempted to characterize these cells by using a panel of mouse monoclonal anti-human cytokeratin antibodies (CK7, 8, 17, 18, and 20) and a rabbit anti-pan cytokeratin serum. Except for the anti-pan cytokeratin serum, which stained ductal epithelia cells in 17.5-dpc pancreas, none of the other cytokeratin monoclonal antibodies stained any ductal cells at 13.5, 15.5, or 17.5 dpc.

Because the dissociated pancreatic cells at 13.5 dpc were cultured in vitro for 4 days (see below), their equivalent stage of development would be ~17.5 dpc in vivo. Therefore, we also characterized the fetal mouse pancreas at this stage. At 17.5 dpc, islet-like structures were formed with β-cells at the core and α-, δ-, and PP-cells at the periphery surrounded by amylase-positive acinar tissues (not shown).

Fetal pancreatic cell culture. To examine cell lineage differentiation, we developed an in vitro serum-free system to culture dissociated fetal pancreatic cells. Hybridoma

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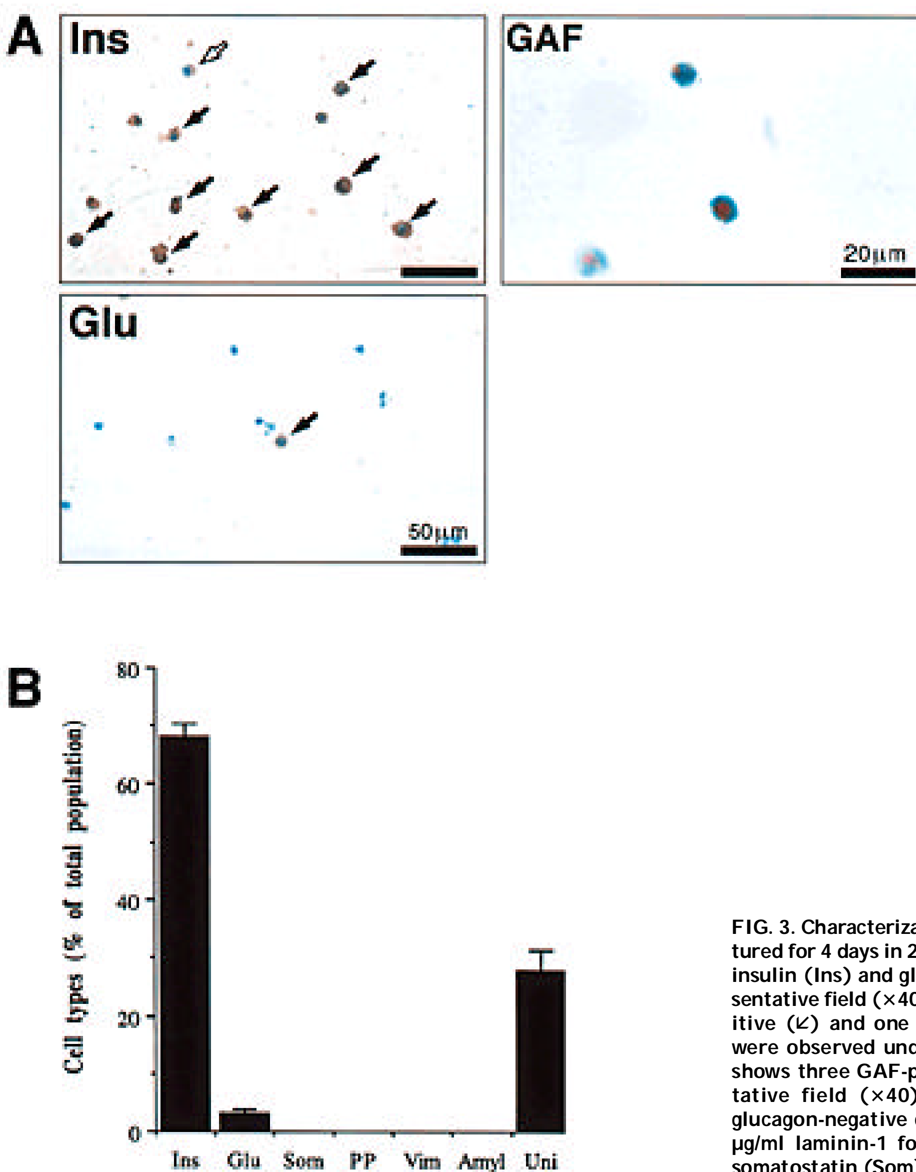

FIG. 3. Characterization of 13.5-dpc fetal mouse pancreatic cells cultured for 4 days in 200 μg/ml laminin-1. *A*: Immunocytochemistry for insulin (Ins) and glucagon (Glu), and staining with GAF. A representative field (×40) was photographed to show mostly insulin-positive (⇙) and one insulin-negative (⇙ open) cells. GAF-stained cells were observed under an oil lens (×100). A representative field shows three GAF-positive cells of various densities. A representative field (×40) shows an α-cell surrounded by numerous glucagon-negative cells. *B*: Quantitation of cells cultured in 200 μg/ml laminin-1 for 4 days after immunostaining for Ins, Glu, somatostatin (Som), pancreatic polypeptide (PP), vimentin (Vim), amylase (Amyl), and unidentified cells (Uni).

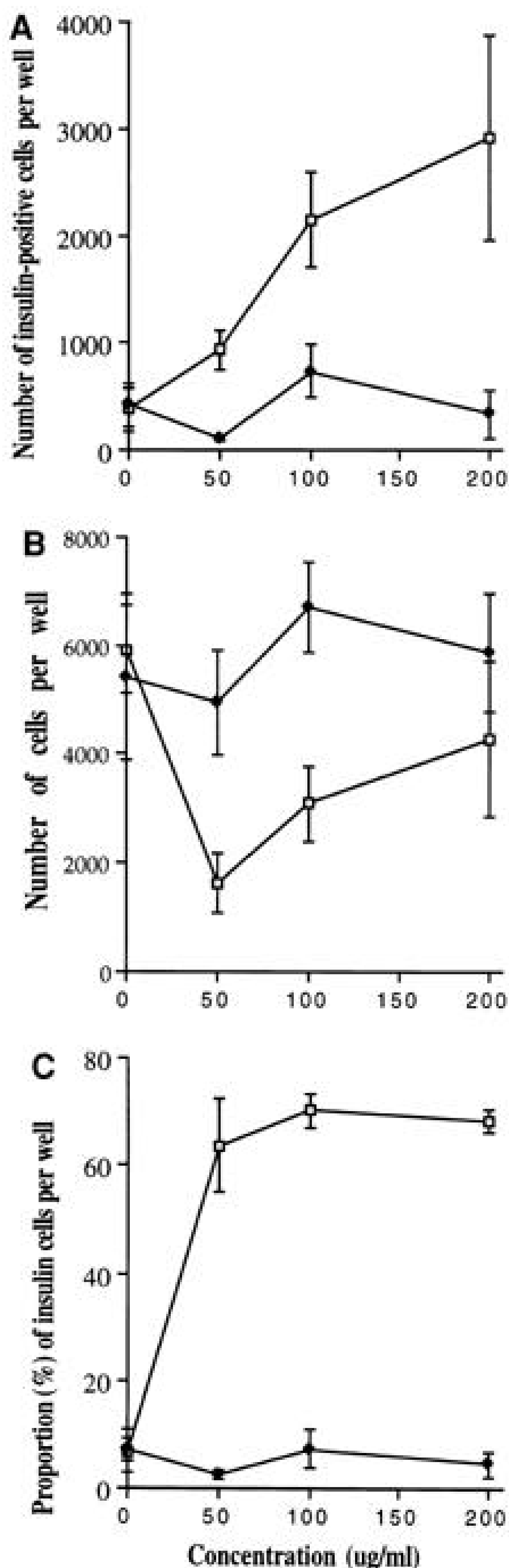

FIG. 4. Dose-response of 13.5-dpc pancreatic cells to laminin-1 (□) and fibronectin (●) during 4 days in culture. *A*: Number of insulin-positive cells; *B*: number of total cells; *C*: proportion of insulin-positive cells in *A*.

medium, a serum-free complete medium originally formulated to replace essential medium supplemented with 10% serum for the growth of hybridoma and lymphoid cells, was found to be suitable for differentiation of these pancreatic cells. The cell density used, 185 cells/mm^2, effectively avoided cell-cell contact and allowed analysis of growth and differentiation at the single cell level. Unlike fibroblasts, which began to extend cytoplasmic processes 2 h after plating and appeared as elongated cells 2 days later, epithelial-like cells maintained a spherical morphology. After 4 days in culture, the total number of cells per well was on average one-third of the number plated, but immunoreactive insulin-positive cells (453 ± 115 per well) were at least 10-fold more numerous than before culture (30 ± 30). Insulin staining was specific because it was absent when guinea pig anti-insulin serum was replaced by preimmune serum and almost completely disappeared after preincubation of anti-insulin serum or monoclonal antibody with excess recombinant human insulin. Thereafter, this culture system was used to test the effect of ECM proteins on 13.5-dpc fetal pancreatic cells.

Fetal pancreatic cell survival in the presence of collagens I and IV. Although the number of β-cells at day 4 of culture was similar at all concentrations of collagen I tested, compared with controls, the total cell number was significantly less

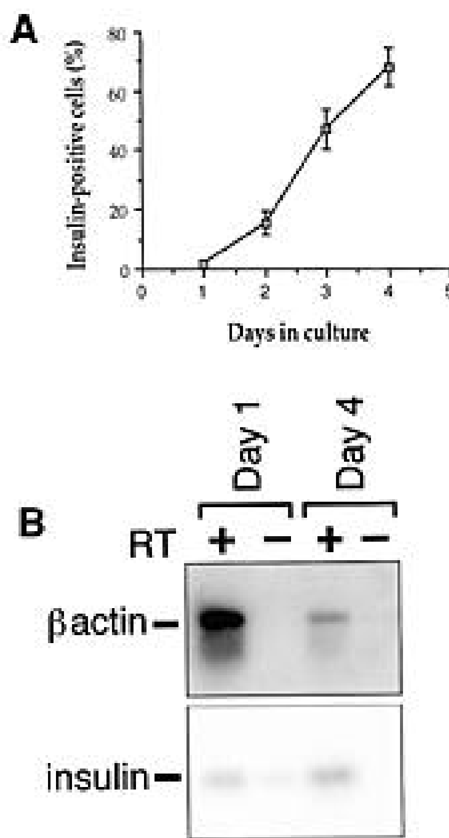

FIG. 5. Increase in insulin-positive cells (*A*) and insulin mRNA (*B*) after culture of 13.5-dpc mouse pancreatic cells for 4 days in 200 μg/ml laminin-1.

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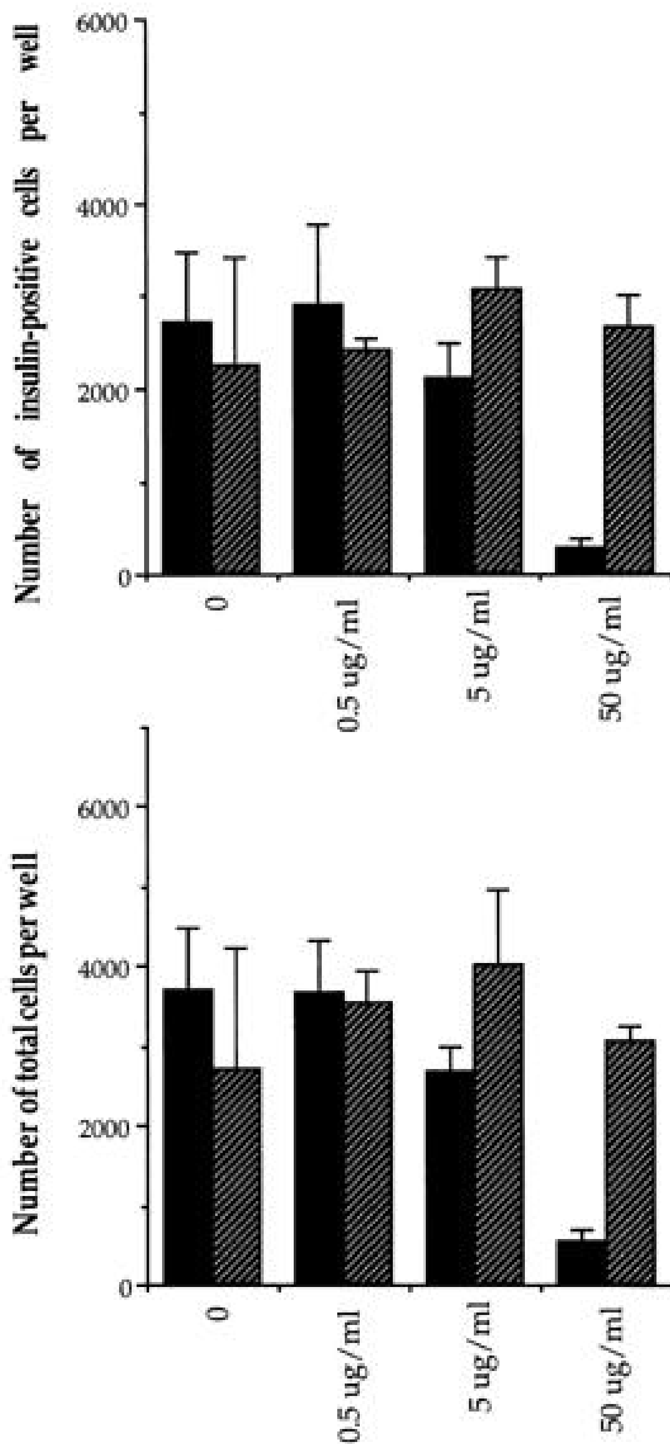

FIG. 6. Effect of anti–laminin-1 antibody (LAM-1) on induction of insulin-positive cells by laminin-1. LAM-1 (0.5–50 μg/ml) was added with 200 μg/ml laminin-1 at the beginning of the 4-day culture. ■, LAM-1; ▨, isotype control.

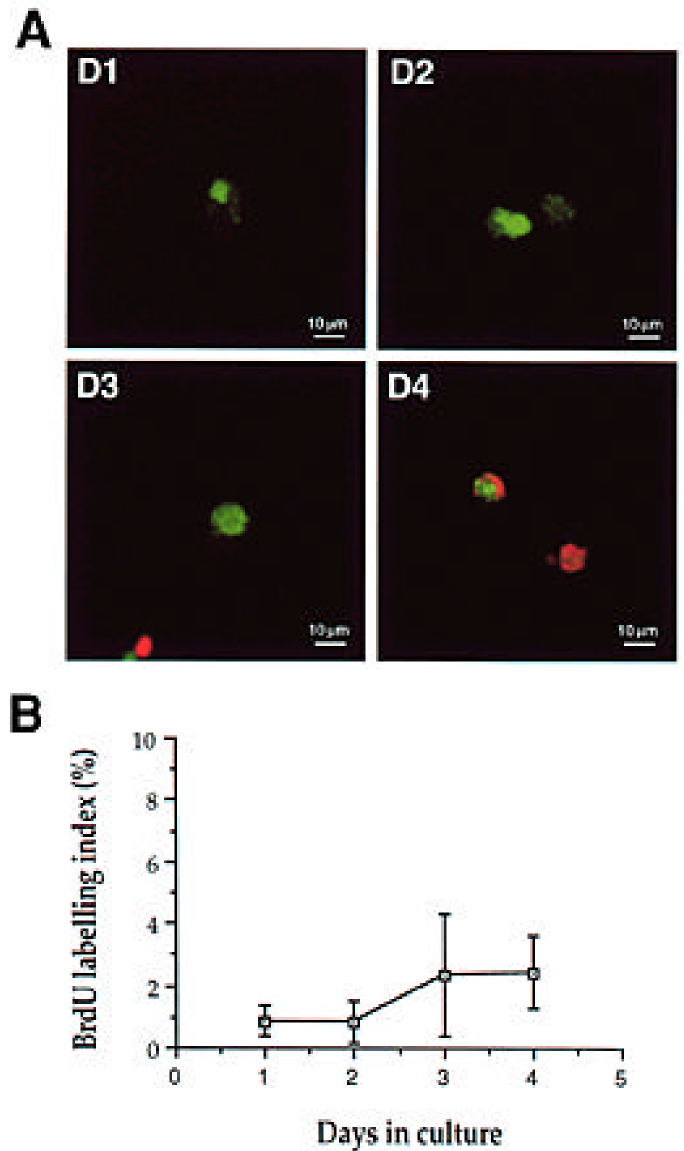

FIG. 7. BrdU labeling of 13.5-dpc fetal mouse pancreatic cells in culture. *A*: Representative photomicrographs showing BrdU-positive cells after double staining for BrdU and insulin. At days 1 (*D1*) and 2 (*D2*) of culture, BrdU-positive cells were all negative for insulin. At day 3 (*D3*), an occasional BrdU-positive cell was insulin-positive. By day 4 (*D4*), all BrdU-positive cells were insulin-positive. *B*: Labeling index of BrdU-positive cells.

($P < 0.05$) in wells coated with 100 and 200 μg/ml collagen I (2,094 ± 162 and 1,379 ± 388) than in those coated with 10 μg/ml collagen I or medium alone (5,516 ± 165 and 4,751 ± 669) (Fig. 2*A*). Similarly, the total number of cells per well was significantly reduced, by 50–80%, after coating with 10–200 μg/ml collagen IV ($P < 0.05$) compared with medium alone (Fig. 2*B*). The inhibitory effect of collagen I was also clearly evident in combination with fibronectin (Fig. 2*C*). Fibronectin alone at 50–200 μg/ml had no obvious effect .

β-Cell differentiation in the presence of laminin-1. After addition of 50–200 μg/ml laminin-1 into the medium, an increased proportion of cultured pancreatic cells were insulin positive (Fig. 3*A*), although their cellular morphology was similar to cells cultured in the absence of laminin-1.

To determine whether insulin-positive cells expressed markers of other cell types, immunostaining for the acinar cell marker, amylase, and for the mesenchymal marker, vimentin, was performed. Neither amylase- nor vimentin-positive cells were detected in chamber slides 4 days after culture in 200 μg/ml laminin-1. To determine whether insulin-positive cells also expressed other hormones, immunocytochemistry for

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glucagon, somatostatin, and pancreatic polypeptide was performed. After 4 days of culture in laminin-1 (200 μg/ml), the number of α-cells (196 ± 15 per well) was similar to that before culture (180 ± 15), while δ- and PP-cells were not detected (Fig. 3*B*). GAF staining was performed to determine whether the insulin-positive cells contained insulin granules characteristic of mature β-cells. Although GAF staining was not intense, cells showed variable GAF positivity (Fig. 3*A*).

The effect of laminin-1 on β-cell number was strongly dose dependent, with a maximum at 200 μg/ml and minimum at 50 μg/ml (Fig. 4*A*). The total cell number was also dose dependent on laminin-1 (Fig. 4*B*). The proportion of insulin-positive cells in cultures containing 50–200 μg/ml laminin-1 was much higher (60–70%) than in medium alone (5%, $P < 0.01$) (Fig. 4*C*). In the presence of 200 μg/ml laminin-1, the proportion of insulin-positive cells increased linearly over 4 days (Fig. 5*A*); up to 5,000 insulin-positive cells per well were observed at day 4 (Fig. 4*A*). RT-PCR was used to compare insulin mRNA expression at days 1 and 4 of culture. By densitometry of band intensities adjusted for cell number using β-actin as a standard, insulin mRNA expression increased between day 1 and day 4. (Fig. 5*B*). Thus, although the total cell number was significantly decreased after 4 days in culture, as indicated by a less-amplified β-actin band, insulin mRNA was increased, consistent with the increase in the number of insulin-positive cells.

To confirm laminin-1 specificity and exclude the possibility that the increase in β-cell number was caused by another component in the laminin-1 preparation, cells were cocultured with 200 μg/ml laminin-1 and the rat monoclonal anti–laminin-1 antibody, LAM-1 (0.5–50 μg/ml). In the presence of 50 μg/ml LAM-1, β-cells were almost completely absent (Fig. 6); there was no obvious effect of lower concentrations of LAM-1 or of an isotype control antibody.

To determine whether the increase of β-cells was due to proliferation of existing β-cells or to differentiation of precursor cells, cultures were incubated with BrdU to label newly synthesized DNA. The presence of insulin-BrdU double-positive cells was analyzed by fluorescent staining at days 1, 2, 3, and 4 of culture. BrdU-labeled cells were mostly non–insulin-positive at days 1–3 (Fig. 7*A*). The maximum labeling index was only ~2.5%, representing incorporation into only ~100 cells over 4 days (Fig. 7*B*). This result indicates that the increase in insulin-positive cells occurs by differentiation of precursor cells, rather than by proliferation of existing β-cells.

Laminin-1 expression in vivo. The effect of laminin-1 in vitro could reflect a role for endogenous laminin in β-cell development. Therefore, we examined the developmental expression of several chains of the laminin family, laminin-α1, -2, and -5, between 13.5 and 17.5 dpc (Table 1). The laminin α1 chain, specific for laminin-1, was detected in the BM of pancreatic ductal epithelium (Fig. 8), but not in the BM of endothelium and smooth muscle (in capsule). The laminin α5 chain was not detected in the epithelial BM of day 13.5–17.5 pancreas, but was expressed in the epithelial BM of the bronchia and kidney tubules at 13.5–17.5 dpc, as reported previously (23), and to varying degrees in stratified squamous and small intestinal epithelia and smooth muscle. The laminin α2 chain was not detected in any of these tissues.

DISCUSSION

Extracellular factors that regulate islet cell lineage differentiation in the pancreas are poorly defined. Studies of these factors have been hampered by the lack of a simple and effective in vitro assay system. We have developed a system based on the culture of limiting numbers of fetal pancreatic cells in a serum-free complete medium. Low cell density was used to avoid any effect of direct cell-cell contact, one of the mechanisms that may regulate cell growth, differentiation, and apoptosis (27). Serum-free complete medium was used because serum may contain >1,000 components, including growth factors and inhibitory substances, which could compromise the interpretation of the studies. The present dissociated cell culture method, therefore, as well as previous organ culture methods (1,15,16), may prove useful for elucidating islet-cell differentiation.

TABLE 1
Expression of laminin-1 and -5 in epithelial basement membrane of different organs analyzed by immunofluorescence staining between 13.5 and 17.5 dpc of development in CBA mice

Epithelial BM	Stage (dpc)	Laminin-1	Laminin-5
Pancreas (ducts)	13.5	+	–
	15.5	++	–
	17.5	++	–
Kidney (tubules)	13.5	++	+
	15.5	++	+
	17.5	++	++
Lung (bronchi)	13.5	–	+
	15.5	+	++
	17.5	–	++
Intestine	13.5	–	–
	15.5	–	–
	17.5	–	+
Skin (stratified squamous)	13.5	–	–
	15.5	–	+
	17.5	–	+

Laminin-1 and -5 were not detected in liver, but the latter was expressed in the BM of liver capsule. Laminin-2 was not detected in any tissue. –, not detected; +, weak to moderate expression; ++, strong expression.

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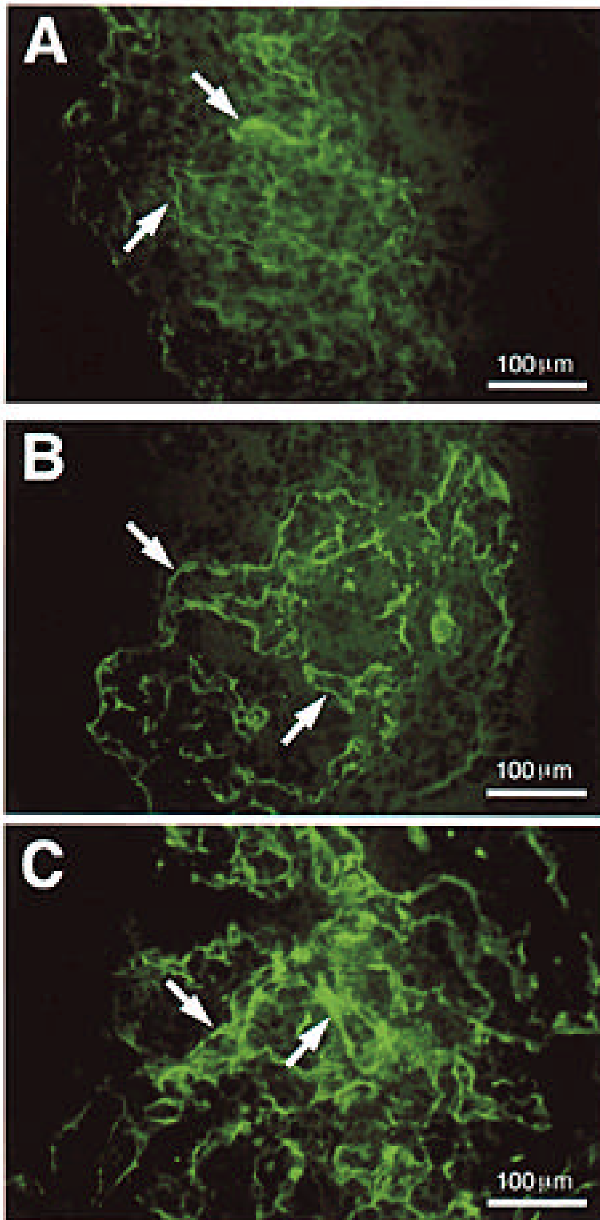

FIG. 8. Expression of laminin-1 in fetal mouse pancreas analyzed by immunofluorescence staining with a rat monoclonal antibody to laminin α1 chain. Laminin-1 expression in the BM ( ) of fetal pancreatic epithelium at 13.5 dpc (*A*), 15.5 dpc (*B*), and 17.5 dpc (*C*) is shown.

Our studies demonstrated that laminin-1 promotes selective differentiation of pancreatic precursor cells into insulin-producing β-cells in vitro. After 4 days of culture in the absence of laminin-1, the total number of surviving pancreatic cells decreased by two-thirds, but the number of insulin-expressing cells increased 10-fold. This appears to represent a significant commitment to β-cell differentiation from pancreatic epithelial cells at 13.5 dpc. In the presence of laminin-1, the number of β-cells increased 60-fold, whereas the number of α-cells remained unchanged and δ-, PP-, and acinar cells were not detected. Thus, pancreatic cellular differentiation in the presence of soluble laminin-1 in vitro differs from that in vivo, where α-, β-, δ-, and PP-cells and acinar cells were all observed 17.5 dpc. However, laminin-1 was specifically expressed in the BM of the fetal pancreatic epithelium, implying that it plays a role in β-cell differentiation in vivo. The effect of laminin-1 was blocked by anti–laminin-1 monoclonal antibody, excluding the possibility that our findings were due to contaminants in the laminin-1 preparation. The upregulation of expression of insulin mRNA in the dissociated pancreatic cells after 4 days of culture in laminin-1 provides additional evidence that these insulin-positive cells are insulin-transcribing β-cells. GAF staining suggests that these β-cells had differentiated to the stage of packaging insulin hexamers into granules characteristics of mature β-cells. BrdU was incorporated into the DNA of only a very small population of non–insulin-positive cells at days 1–3 that were insulin-positive cells at day 4, consistent with the idea that insulin-positive cells developed predominantly by differentiation from precursor cells in day-13.5 fetal pancreas. It is not clear why the total cell number decreased in the presence of laminin-1. In the presence of laminin-1, a subpopulation of cells may selectively adhere to the culture plate, although adherence per se is unlikely to account for the differentiation effect. Many primary and transformed cells need to adhere for survival and proliferation. We are not aware of evidence, however, that attachment per se induces substantial differentiation. On the contrary, attachment of many types of cells induces dedifferentiation, not differentiation. Expression of laminin-1 in the BM of the fetal pancreatic epithelium implies that epithelial cells, not fibroblasts, express receptors for laminin-1. It is likely that laminin-1 selectively binds to precursor epithelial cells to induce changes that then enable them to attach and differentiate.

Laminin-1 plays an important role in proliferation and differentiation of many cell types during development (28). It is a heterotrimeric glycoprotein (relative molecular weight = 850,000) composed of α (400 kDa), β (210 kDa), and γ (200 kDa) disulfide-bonded chains (28,29). More than 10 different laminin chains have been cloned, including α1–5, β1–3, and γ1–2, and it is assumed that 11 different isoforms may exist (30). Laminin-1 is the earliest described and most extensively studied member of this family. By electron microscopy, the structure of laminin-1 was shown to be cruciform, with one long and three short arms. The three similar short arms are contributed by the NH_2-terminal globular regions of the α1, β1, and γ1 chains, whereas the long arm is formed by the three chains, linked together by disulfide bonds (13). Laminin-1 (α1β1γ1) is expressed by many embryonic epithelial cells (28) and has been shown to induce specifically β-casein gene expression in mammary epithelia (31) and neuron generation from retinal neuroepithelial cells (32). The cross region of laminin-1 selectively promotes fetal lung epithelial cell proliferation; the outer globular region of the α1 and β1 chains mediates epithelial cell polarization; and the inner globular region of the β1 chain binds to heparin sulfate proteoglycan and stimulates lumen formation (29,33). There are at least two families of laminin-1 receptors: the integrin family and the non-integrin family (14). The spatial and temporal expression of these receptors during pancreatic development and their involvement in signaling the laminin stimulus into cytoplasma and nucleus are currently under investigation. It remains to be defined how laminin-1 binds to receptors on fetal pancreatic epithelial cells; as with receptors on other embryonic epithelium, binding possibly occurs to the globular domains at the COOH-terminal of the α-chain.

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Recent evidence indicates that integrin-mediated cell-matrix interactions can induce apoptosis in epithelial and endothelial cells (34). The dramatic decrease in survival of dissociated pancreatic cells plated onto collagens I and IV suggests that certain ECM-integrin interactions may activate apoptotic signaling pathways. These results are similar to those of Boudreau et al. (35), who showed that the mammary epithelial cell line CID-9 underwent apoptosis on a collagen matrix and proposed that this was due to an integrin-dependent negative regulation of expression of interleukin-1β converting enzyme (ICE).

In conclusion, we demonstrate that laminin-1 specifically induces differentiation of β-cells in serum-free culture of fetal mouse pancreatic cells. The expression of laminin-1 in fetal pancreatic ductal epithelium indicates it is likely to be involved in promoting differentiation of β-cells in vivo.

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