Expression and Role of Laminin-1 in Mouse Pancreatic Organogenesis

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Previous studies have suggested that basement membrane alone may induce ductal differentiation and morphogenesis in the undifferentiated embryonic pancreas. The mechanism by which this induction occurs has not been investigated. Studies of other organ systems such as the lungs and mammary glands, where differentiation has been shown to be induced by basement membrane, have suggested a major role for laminin as a mediator of ductal or tubular morphogenesis and differentiation. We first defined the ontogeny of laminin-1 in the developing mouse pancreas. To determine the specific role of basement membrane laminin in pancreatic ductal morphogenesis and differentiation, we microdissected 11-day mouse embryonic pancreatic epithelium free from its surrounding mesenchyme and then suspended the explants in a 3-dimensional organ culture to allow us to assay cell differentiation and morphogenesis. When the pancreatic epithelium buds off the foregut endoderm, the pancreatic mesenchyme diffusely expresses laminin-1. This laminin subsequently organizes to the interface between the epithelium and the mesenchyme by E12.5. As gestation progresses, epithelial cells in direct contact with laminin-1 seem to differentiate into ducts and acini, whereas those spared intimate contact with laminin-1 appeared to organize into islets. Although basement membrane gel could induce pancreatic ductal morphogenesis of embryonic pancreatic epithelium, this induction was blocked when we added neutralizing antibodies against any of the following: 1) laminin (specifically laminin-1), 2) the “cross-region” of laminin-1, and 3) the α6 moiety of the integrin receptor, which is known to bind laminins. Immunohistochemistry, however, showed that pancreatic duct cell-specific differentiation (carbonic anhydrase II) without ductal morphogenesis was still present, despite the blockage of duct morphogenesis by the anti-laminin-1 neutralizing antibodies. Interestingly, there appeared to be a decrease in carbonic anhydrase II expression over time when the epithelia were grown in a collagen gel, rather than in a basement membrane gel. The pattern of laminin-1 expression in the embryonic pancreas supports the conclusion that laminin-1 is important in the induction of exocrine (ducts and acini) differentiation in the pancreas. Furthermore, our data demonstrate that 1) pancreatic ductal morphogenesis appears to require basement membrane laminin-1 and an α6-containing integrin receptor; 2) the cross-region of basement membrane laminin is a biologically active locus of the laminin molecule necessary for pancreatic ductal morphogenesis; 3) duct-specific cytodifferentiation, in the form of carbonic anhydrase II expression, is not necessarily coupled to duct morphogenesis; and 4) the basement membrane gel may contain components (e.g., growth factors) other than laminin-1 that can sustain both carbonic anhydrase II expression and, possibly, the capacity to form ducts, despite the absence of duct structures. Diabetes 49:936–944, 2000

Organogenesis involves 2 processes: morphogenesis, which is the shaping of an organ, and cytodifferentiation, which is the acquisition and expression of specialized cellular functions within that organ (1,2). The mechanisms through which these 2 processes of organogenesis are induced and regulated are not fully understood. Studies in the 1960s and 1970s supported the idea that interactions between pancreatic epithelium and mesenchyme are necessary for organogenesis to occur (1,3–7). Research in the 1970s and 1980s tried to establish the nature of the control of pancreatic mesenchyme over the pancreatic epithelium in pancreatic organogenesis (5,8–11). For a long time, it was thought that pancreatic organogenesis was exclusively under the control of mesenchyme and that without mesenchyme, complete organogenesis into endocrine islets and exocrine ducts and acini would not occur. We previously investigated the role of mesenchyme in pancreatic organogenesis. We found that the presence of pancreatic mesenchyme was necessary only for pancreatic acinar differentiation. Mature islet differentiation, however, occurred in the absence of mesenchyme if the isolated pancreatic epithelium was grown under the renal capsule of an adult mouse. In addition, pancreatic ductal morphogenesis and cytodifferentiation occurred in vitro in a basement membrane–rich gel (12).

It has been well established that basement membrane, which forms at the interface between epithelium and mesenchyme, plays a key role in epithelial-mesenchymal interactions in numerous developing mammalian organ systems (13–24). Basement membrane is variably made up of collagens, proteoglycans such as heparan sulfate and chondroitin sulfate, and noncollagenous glycoproteins such as laminin and fibronectin (17,25–30). Several of these basement membrane components have been shown to be biologically active in organogenesis (13–24,31–35).
Laminin is the major noncollagenous protein in basement membrane, and its role in both morphogenesis and cytodifferentiation has been well established. Laminin has been shown to mediate cell-cell and cell-substrate adhesion, cell movement, and cell proliferation (14,15,25,36–40). These processes are thought to be crucial for normal morphogenesis to occur. Laminin mediates branching morphogenesis in the lung and the salivary gland, and laminin induces cytodifferentiation in the developing liver and breast (14,19, 22–24,31,41–50).

In general, laminins are multidomain glycoproteins, which are found predominantly in basement membrane. They are cross-shaped heterotrimeric molecules composed of 3 polypeptide chains (α, β, and γ), which are linked to one another by disulfide bonds (51). At least 10 different isoforms of laminin, made up of various combinations of the 5 known α-chains (α1–α5), the 3 different β-chains (β1–β3), and the 2 different γ-chains (γ1 and γ2), have been defined. Laminin-1 (α1β1γ1), which is the predominant glycoprotein in basement membrane, has been the most commonly studied form of laminin, and most analyses of development thus far have been based on laminin-1. Laminin-1 is usually studied by tracing the α1 chain of laminin or by using polyclonal antibodies specifically against laminin-1 (15,25).

In the case of the pancreas, although it is known that a basement membrane–rich gel alone can induce ductal morphogenesis and cytodifferentiation from undifferentiated pancreatic mesenchyme, it is not known what roles, if any, laminins may play in this induction. Furthermore, it is unkown if pancreatic ductal morphogenesis and cytodifferentiation are induced by the same mechanism. To begin to investigate these questions, we first examined the ontogeny of laminin-1 in the embryonic mouse pancreas. We then grew early embryonic pancreatic epithelia in various 3-dimensional organ cultures to assay for morphogenesis and cytodifferentiation histologically and by immunohistochemistry.

RESEARCH DESIGN AND METHODS

Animals. Time-dated pregnant CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were killed on days 10.5, 11.5, 12.5, 15.5, and 18.5 of gestation (day 0.5 defined as the morning of discovery of vaginal plug). Microdissection was performed on the individual embryos to isolate and remove the pancreases as previously described (12). E11.5 pancreases were fixed in the paraformaldehyde for 2 h and then transferred to 30% sucrose and families. E10.5 pancreases were cultured in 5% CO2 at 37° C for 3–8 days, during which time they were harvested and incubated (0.5% in PBS) wash. For immunofluorescent double staining, the slides were also subjected to 150 µl of a collagen I gel (Vitrogen; Collagen Biomedical, Palo Alto, CA) alone, a basement membrane–rich gel (Matrigel; Collaborative Research, Boston, MA) alone, or basement membrane–rich gel incubated with 1 of the following: polyclonal anti-laminin antibody (donkey anti-human laminin-1; Accurate Chemical and Scientific, Westbury, NY), polyclonal anti-laminin antibody preincubated with 200 µg/ml free laminin-1 (Collaborative Research) for 30 min, monoclonal anti-laminin antibodies directed against each of 5 different domains of the laminin-1 molecule (rat anti-mouse; Chemicon, Temecula, CA), or monoclonal antibodies against different integrin moieties known to bind laminins, including α1, α3, α5, and α6 integrin receptors (rat anti-human; PharMingen, San Diego, CA). For each antibody, different concentrations were used initially, and subsequent studies were performed at a 1:5 dilution based on maximal effects at that dilution.

In addition, as a control, epithelia were grown in collagen gels for 7 days, either with or without added exogenous laminin-1 at concentrations ranging from 5 ng/ml to 100 µg/ml (Collaborative Research). Isolated embryonic pancreatic epithelia were transferred into the various gels before gel polymerization, using sterile siliconized tips under direct microscopic vision. Tissues were cultured in 5% CO2 at 37°C for 3–8 days, during which time they were harvested for microscopic and immunohistochemical analysis. At least 10 successful cultures (i.e., free of technical problems or infection) were used for each experimental group, and minimal variability was seen in these groups.

RESULTS

Our examination of the ontogony of laminin-1 in the embryonic mouse pancreas demonstrated an intimate relationship between laminin-1 and the developing exocrine pancreas. At E10.5, soon after the pancreas evaginates from the foregut endoderm into the pancreatic mesenchyme, laminin-1 protein was detected exclusively within the mesenchymal cells, with no staining in the epithelium (Fig. 1). By E12.5, an extracellular deposition of laminin-1 was apparent, forming a rim around the developing epithelial cells. This rim was at the interface between the epithelium and the mesenchyme. This gestational time point corresponds to the early expression of acinar genes (12,52). By E15.5, maturing acinar structures were evident. Laminin-1 expression was tightly associated with the acini, forming a rim around each developing acinus. Laminin-1 was also strongly expressed along the basal surface of the developing ducts. This pattern matured at E18.5, at which point we saw a strong association of laminin-1 expression rimming the mature acini and adjacent to the ducal epithelium.

At E15.5, individual cells within the ducal epithelium stained positively for insulin. Additionally, groups of cells that were spared intimate contact with laminin-1 grew before overnight exposure to the primary antibody at 4°C, followed by secondary antibody (biotinylated anti-rabbit immunoglobulin [Vectastain; Vector Laboratories, Burlingame, CA]) for 1 h. The slides were then exposed to the avidin-biotin-peroxidase solution (Vectastain; Vector Labs) for 1 h. The immunohistochemical reaction was completed with exposure of the tissues to 3,3-diamino-benzidine. Finally, the tissue sections were counterstained with Mayer's Hematoxylin (Sigma) and saturated Li2CO3.

For immunofluorescent double staining, the slides were also subjected to treatment with sodium citrate and Tween-20 (0.5% in PBS). Incubation with the blocking serum (normal donkey serum in PBS 1:25) took place overnight at 4°C. The primary antibodies were applied together for 3 h at 25°C after another exposure to Tween-20 (0.5% in PBS). Finally, the fluorescent secondary antibodies (donkey anti-sheep tetramethylrhodamine isothiocyanate 1:200 and donkey anti-rabbit fluorescin isothiocyanate 1:200) were added after 1 more Tween-20 (0.5% in PBS) wash.

Pancreas organ culture. Isolated E11.5 whole pancreases, or pancreatic isolated epithelia (free of mesenchyme), were grown under various culture conditions in filter inserts (Millipore, Bedford, MA) and placed in standard 24-well plates under sterile conditions. Each well was filled with 500 µl filter-sterilized Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) and 1% antibiotic/antimycotic solution (10,000 U/ml penicillin G, 10,000 µg/ml streptomycin sulfate, and 20 µg/ml amphotericin B [Gibco]). Filter inserts were filled with either 150 µl of a collagen I gel (Vitrogen; Collagen Biomedical, Palo Alto, CA) alone, a basement membrane–rich gel (Matrigel; Collaborative Research, Boston, MA) alone, or basement membrane–rich gel incubated with 1 of the following: polyclonal anti-laminin antibody (rabbit anti-human laminin-1; Accurate Chemical and Scientific, Westbury NY), polyclonal anti-laminin antibody preincubated with 200 µg/ml free laminin-1 (Collaborative Research) for 30 min, monoclonal anti-laminin antibodies directed against each of 5 different domains of the laminin-1 molecule (rat anti-mouse; Chemicon, Temecula, CA), or monoclonal antibodies against different integrin moieties known to bind laminins, including α1, α3, α5, and α6 integrin receptors (rat anti-human; PharMingen, San Diego, CA). For each antibody, different concentrations were used initially, and subsequent studies were performed at a 1:5 dilution based on maximal effects at that dilution.
FIG. 1. Immunohistochemistry. A: Schematic representation of E10.5 whole pancreas (shown histologically in B). The epithelium (black), surrounded by mesenchyme (gray), forms lobulations among which the mesenchyme interdigitates, as indicated by the arrows. The mesenchymal island (••) in the middle of the epithelium represents another interdigitation (as shown in cross-section). B: At E10.5, laminin-1 staining (brown) localizes specifically to the pancreatic mesenchyme. The epithelium is spared. Brown-staining mesenchyme interdigitates between epithelial lobules, as indicated by the arrows and arrowheads. Original magnification ×200. C: At E11.5, laminin-1 (brown) begins to localize to the interface, as indicated by the arrows, between the epithelium and the mesenchyme. Numerous mesenchymal cells continue to express laminin-1 (►). Original magnification ×400. D: At E12.5, the pancreatic mesenchyme still expresses laminin-1 (►). Additionally, laminin-1 staining localizes strongly to the interface, as indicated by the arrows, between the epithelium and the mesenchyme. Original magnification ×400. E: At E15.5, laminin-1 staining, as indicated by the arrows, is intimately associated with the exocrine pancreas. Laminin-1 staining is visible around all of the developing acini and at the basal surface of ductal epithelial cells. Original magnification ×200. F: At E15.5, insulin-stained cells, as indicated by the arrows, appear sporadically along the developing ducts. Original magnification ×400. G: At E18.5, laminin-1 staining, as indicated by the arrows, is still visible around all acini and is adjacent to ductal epithelial cells. Interestingly, cells that lack intimate contact with laminin-1 (at the tips of the ducts) grow together into islet clusters. Laminin-1 ensheaths these maturing islets. Original magnification ×200. H: At E18.5, the cells that were spared intimate contact with laminin-1 (E) stain specifically for insulin (brown) and begin to take the shape of islets. Original magnification ×200. I: Double-immunofluorescent staining for laminin-1 (green) and insulin (red) at E18.5. Within the developing islets, laminin-1 is only expressed in what appear to be new blood vessels, as indicated by the arrows. The cluster of endocrine cells is essentially spared contact with laminin-1. In the adjacent duct, laminin-1 surrounds the ductal epithelium. Original magnification ×400. A, acini; D, duct; E, epithelium; I, islets; M, mesenchyme.
together into discrete clusters. These clusters were readily apparent by E18.5. It became clear that they represented nascent islets with strong insulin staining. Within the developing islet clusters, laminin-1 expression was only seen sparingly, in what appeared to be developing blood vessels. These results were confirmed with double-immunofluorescent staining for insulin and laminin-1 (Fig. 1I). The mature islets in the late gestational pancreas did become ensheathed by a rim of laminin-1 (Fig. 1G). This proximity of laminin-1 to the islets occurs after commitment to the endocrine lineage and thus may play a secondary role, possibly to organize islet architecture.

To corroborate the aforementioned in vivo data, E11.5 pancreases were cultured for 5 days in a collagen gel, and immunohistochemistry was again performed for laminin-1. Here, the patterns of laminin-1 and insulin staining were similar to the fresh embryonic pancreas explants (Fig. 2). There was a clear polarization of insulin staining to groups of cells that lacked intimate contact with laminin-1. Laminin-1 appeared to be expressed only in the exocrine component of the cultured pancreas.

After defining the spatial and temporal association of laminin-1 expression with the developing exocrine pancreas, we sought to determine the specific role of laminin-1 in pancreatic ductal morphogenesis and cytodifferentiation. We grew isolated embryonic pancreatic epithelia in different culture systems. Initially, the E11.5 pancreatic epithelia grown on a basement membrane–rich gel consistently formed multiple cystic structures. We have previously shown that these cysts represent ductal tissue based on duct-specific immunohistochemistry and electron microscopy (12). We then added neutralizing polyclonal antibodies specific for laminin-1 to isolated pancreatic epithelium grown in basement membrane gel. Isolated pancreatic epithelia that were grown in basement membrane–rich gels incubated with this polyclonal anti-laminin-1 antibody at a 1:5 dilution consistently failed to form ductal structures (Fig. 3A).

To further establish that laminin-1 is necessary for duct formation, we attempted 3 confirmatory experiments. First, to be sure that the blockage of duct formation with the polyclonal anti-laminin antibodies was a specific effect, we grew the isolated pancreatic epithelia in a basement membrane–rich gel incubated with polyclonal anti-laminin-1 antibodies (1:5 dilution). Note the absence of cystic structures, which is indicative of a blocking effect of the antibodies. B: Isolated epithelium after 8 days in a basement membrane–rich gel with nonimmune serum. The presence of cystic structures suggests that the addition of antibodies does not have a nonspecific effect on the induction of pancreatic ductal morphogenesis by basement membrane. C: Isolated epithelium after 8 days in a basement membrane–rich gel with polyclonal anti-laminin-1 antibodies (1:5 dilution) that were first preincubated with exogenous laminin-1 (200 µg/ml) for 30 min. The formation of multiple cystic structures further implicates a specific role for laminin-1 in pancreatic ductal morphogenesis and confirms the specific effects of the antibodies. D: Isolated epithelium after 8 days in a basement membrane–rich gel with neutralizing anti-α6 integrin receptor antibodies (1:5 dilution). The lack of cystic structures suggests that the mechanism by which laminin-1 induces pancreatic ductal morphogenesis is via an interaction with the α6-containing integrin. Original magnification ×100. E: Isolated epithelium after 8 days in a basement membrane–rich gel with monoclonal antibodies (1:5 dilution) directed against the laminin-1 cross-region. Note the absence of cystic morphology. Monoclonal antibodies directed against 4 other regions of laminin-1 consistently failed to inhibit cystic formation. Original magnification ×100.
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mation (Fig. 3D). The α6-containing integrin receptor is a cell membrane receptor known to bind laminins (37,53).

Finally, having established that laminin-1 is a necessary component in inducing ductal morphology from undifferentiated embryonic pancreatic epithelium, we next attempted to identify the exact region of the laminin-1 molecule that was responsible for this induction. We grew isolated embryonic pancreatic epithelia on a basement membrane–rich gel incubated separately with each of the 5 different monoclonal anti-laminin antibodies. Each monoclonal anti-laminin antibody recognized a separate domain of the laminin heterotrimer. After 7 days in culture, only the antibody that recognized the specific cross-region of the laminin-1 molecule consistently blocked duct formation (Fig. 3E). Control experiments were also performed using nonimmune rabbit and mouse serum. These sera had no effect on the ability of the pancreatic epithelium to form ducts.

In all of our organ culture experiments, we also wished to determine the relationship between the morphogenesis of duct-like structures and the expression of markers of cytodifferentiation. We used carbonic anhydrase II expression, as detected by immunohistochemistry, as an indicator of duct-cell differentiation. Embryonic pancreatic epithelia grown in basement membrane gel with added antibodies consistently stained positively for carbonic anhydrase II, regardless of morphology (Fig. 4). The relative level of intensity was not measured quantitatively, and the number of cells expressing carbonic anhydrase II was not precisely quantified in the 2 groups. However, there were clearly numerous positive cells in the epithelia that did not form ducts. Thus, the expression of this duct-specific marker occurred independently of the morphogenic formation of duct-like structures.

To better understand the relationship between duct morphogenesis and carbonic anhydrase II expression, we studied the expression of carbonic anhydrase II at the time when the cultures were initiated (i.e., in the fresh E11.5 embryonic mouse pancreas). Here, we found small clusters of carbonic anhydrase II–expressing cells (Fig. 5), despite the absence of any detectable duct-like structures. We interpreted this population of cells to be early cells in the developing pancreas with the potential to form ducts. Next, we studied the expression of this marker over time in epithelia grown in the basement membrane–rich gel. After 2 days in the basement membrane gel culture, there were carbonic anhydrase II–positive cells that did not appear to be part of forming ducts (Fig. 6A and B). At this early 2-day time point, there were no obvious duct-like structures. By 5 days, in culture in basement membrane gel, duct structures were beginning to form. These duct structures were positive for carbonic anhydrase II, but there was still evidence of carbonic anhydrase II–positive cells located centrally in the growing pancreatic epithelia that did not seem to be forming ducts (Fig. 6C and D). These cells were confirmed not to be tangential cuts on cystic structures by performing serial section analysis. These results are again consistent with the concept that

FIG. 4. Immunohistochemistry for carbonic anhydrase II, a marker for pancreatic ductal differentiation. A and B: Staining of isolated epithelium after 8 days in a basement membrane–rich gel alone. Note the cystic morphology. The specific carbonic anhydrase II staining (brown) indicates ductal differentiation. The sections are counterstained with hematoxylin. Original magnifications ×200 and ×400, respectively. As shown in B, there are a few positive stained cells that are not part of the duct structure, but the predominant positive staining is localized to the duct structure. Original magnification ×400. C: Staining of isolated epithelium after 8 days in a basement membrane–rich gel with monoclonal anti-laminin-1 antibodies (1:5 dilution) directed against the laminin-1 cross-region. Note that the presence of cystic morphology is minimal. Carbonic anhydrase II staining is present in regions where ductal morphogenesis is beginning, as indicated by the arrows, and in regions with no evidence of ductal morphology (►). Original magnification ×400.

FIG. 5. Immunohistochemical staining of E11.5 pancreas immediately after harvesting. There are 2 clusters of cells that stain for carbonic anhydrase II. These cells may represent early duct precursor cells. Original magnification ×400. E, epithelium; M, mesenchyme.
duct-specific marker expression is not obligately coupled to the morphogenesis of ducts.

Lastly, to study the possibility that other factors in the basement membrane gel may contribute to expression of the duct marker, we analyzed the expression of carbonic anhydrase II in isolated pancreatic epithelium grown in a collagen gel for 7 days. These epithelia showed minimal expression of carbonic anhydrase II (Fig. 7). This decrease in expression, when compared with the basement membrane gel cultures or even with the fresh E11.5 pancreas, suggests that factors in the basement membrane gel are necessary to support the isolated epithelial expression of carbonic anhydrase II. This basement membrane gel is known to contain significant quantities of many growth factors, and 1 or more of these factors may be playing a role in supporting carbonic anhydrase II expression.

**DISCUSSION**

Early studies establishing the differentiation of embryonic pancreatic epithelium during organogenesis is under the influence and control of regional mesenchyme (1,3–7) served as the foundation for subsequent work that suggested a specific role for basement membrane in pancreatic development. Basement membrane alone was shown to be sufficient for the induction of pancreatic ductal morphogenesis (12). This finding is consistent with the fact that basement membrane, which forms at the interface between epithelium and mesenchyme, has been shown to play a major role in certain aspects of epithelial differentiation in numerous organ systems (13,15,18,19,25,54).

The specific components of basement membrane that may be necessary for pancreatic duct formation have never been studied. In this study, we investigated the role of laminin-1, which is a major component of basement membrane, in inducing pancreatic ductal morphogenesis from undifferentiated precursors. We also studied the relationship between duct formation and the expression of a duct-specific marker, carbonic anhydrase II. From our results, we conclude that basement membrane laminin-1, possibly through an interaction between its cross-region and an α6-containing integrin, is necessary for ductal morphogenesis. Pancreatic ductal cyt differentiate, however, may occur independent of laminin-1 signaling.

Laminin-1 is expressed throughout the pancreatic mesenchyme, into which the pancreatic epithelium grows after it has evaginated from the foregut endoderm. Between E10.5 and E12.5, the mesenchymal laminin-1 begins to organize at the basement membrane at the interface of the growing epithelium and the mesenchyme. The mechanism of recruitment, organization, and concentration of diffuse laminin into an organized lattice adjacent to epithelium has recently been elucidated. This process appears to be mediated by α-dystroglycan (27). Organization of laminin is the critical first step for basement membrane development (27).

Laminin-1 is often limited to the true epithelial basement membrane of organs and in fact has been found to encompass the entire basal surface of the exocrine structures of the adult pancreas (15,55). We found that as pancreatic organogenesis progresses, those epithelial cells that maintain intimate contact with laminin-1 appear to differentiate into exocrine struc-

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**FIG. 6.** Immunohistochemical staining for carbonic anhydrase II in isolated pancreatic epithelium grown for either 2 days (A and B) or 5 days (C and D) in a basement membrane–rich gel, showing the time course of expression of the duct mark. A: At day 2 in culture, 2 clear clusters of cells that are weakly positive for carbonic anhydrase II are visible, as indicated by the arrows. These cells may represent cells similar to those shown in the freshly harvested E11.5 pancreas in Fig. 5. Original magnification ×200. B: Higher magnification (×400) of carbonic anhydrase II–expressing cells in the pancreatic epithelium after 2 days in Matrigel. These cells may be organizing into a duct. C: After 5 days in culture, multiple ducts expressing carbonic anhydrase II, as indicated by the arrows, have been formed by the pancreatic epithelium. Original magnification ×200. D: Higher magnification (×400) of pancreatic ducts expressing carbonic anhydrase II after the epithelium has been in culture for 5 days. Mg, Matrigel.

**FIG. 7.** Immunohistochemical staining for carbonic anhydrase II of isolated pancreatic epithelium grown in a collagen gel, rather than a basement membrane–rich gel (Matrigel), for 5 days. The arrows indicate a couple of the cells that continue to express carbonic anhydrase II. Overall, the number of cells expressing carbonic anhydrase II appears to be much lower than that seen when the E11.5 pancreatic epithelium was grown for 5 days in Matrigel with or without anti-laminin antibodies (Figs. 4 and 6). By day 7, there are no detectable carbonic anhydrase II–expressing cells in the pancreatic epithelium grown in a collagen gel (data not shown). Original magnification ×400.
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tures (i.e., ductal epithelial cells and acinar cells). In contrast, the cells that are spared direct contact with laminin-1 appear to organize into discrete clusters of cells that express endocrine markers. The clusters proceed to form islets by the end of gestation (E 18.5). These observations in vivo are consistent with our previous findings that basement membrane has the capacity to induce ductal morphogenesis in early pancreatic epithelia, whereas the absence of such induction (when the early epithelia are grown under the renal capsule there may be inadequate contact with laminin-1, especially on the capsular side) allowed for a default selection of endocrine lineage (12). The ensheathment by laminin-1 of the mature islets at E 18.5 apparently cannot influence or rescue the differentiated cells within the islet from their commitment to the endocrine lineage.

Our laminin-1 ontogeny data and our embryonic pancreas organ culture experiment, in which the laminin-1 expression is polarized away from the endocrine cells, would seem to argue against a biological role for laminin-1 in endocrine differentiation. This assertion is in direct contrast to data published by Jiang et al. (56), in which β-cell differentiation is purported to be induced by laminin-1. That conclusion is based on a unique experimental system in which embryonic pancreatic cells are dispersed and then cultured with laminin or collagen (56). The enzymatic and mechanical dissociation of the pancreatic cells may alter their behavior. Furthermore, islets have been shown to contain predominantly the α 3 β 1 integrin, which does not bind laminin-1 (57). We deduced from the data compiled by Jiang et al. (56) that stem cells of the pancreas initially require laminin-1 contact to allow later endocrine differentiation when laminin-1 is removed.

Our organ culture studies all suggest that laminin-1 is specifically required for pancreatic duct development. The interactions of the respective laminin components with cell surface receptors are known to evoke responses that are specific to each cell type. For example, Schuger et al. (43) revealed that 2 laminin epitopes, the cross-region, and the globular regions of the β-chain play unique roles in lung development. Our study found a role only for the laminin cross-region in pancreatic ductal morphogenesis, yet specific activities such as cell attachment and migration were not assayed because no representative cell line or monolayer is currently available.

Although our data suggest that the laminin-1 cross-region mediates pancreatic ductal morphogenesis, possibly via an α 6 -containing integrin, prior studies have found that α 6 binds to the base of the laminin α-chain, not the cross-region (53). Although our data support the existence of an interaction between the laminin cross-region and an α 6 -containing integrin, we can draw no conclusions about the exact nature of this interaction or whether a direct interaction occurs. It is possible that other factors, like conformational changes and steric interactions, may be involved (58). It is also possible that the integrin has a role independent of the cross-region and that it only serves to stabilize the adhesion of the epithelium to the basement membrane. The role of α 6 in pancreatic duct formation is consistent with the finding of α 6 -containing integrins in the pancreas and in pancreatic ductal cancers (59–62).

Our laminin isoforms may also play a role in pancreatic duct formation, and significant blockade by these antibodies of other laminins or by laminin-associated molecules, such as nidogen, collagen IV, or dystroglycan, are also possible. However, the blocking effect of the antibody used in these experiments appeared to be specific, as evidenced by the fact that preincubation of the antibody with exogenous laminin-1 reversed the effect. These results do not absolutely rule out the possibility of cross-reactivity of the neutralizing antibody to another molecule that is critical for duct formation, but such a possibility seems unlikely.

Even as these data concerning neutralizing antibodies suggest that laminin-1 and the α 6 -containing integrin may be necessary for pancreatic ductal morphogenesis, no such assertions can be made with respect to pancreatic ductal cytodifferentiation. Using carbonic anhydrase II as a marker of duct-cell differentiation, we found very little evidence of a relationship between the spatiotemporal expression of carbonic anhydrase II in the developing pancreas and the morphogenesis of ducts from the embryonic pancreas. The ducts that formed always stained strongly for carbonic anhydrase II, but, surprisingly, carbonic anhydrase II was also detected in discrete clusters of cells in the early (E 11.5) pancreas, before any evidence of duct formation (Fig. 5). Interestingly, when these E 11.5 epithelia were grown in a collagen gel, instead of a basement membrane gel, the carbonic anhydrase II expression seemed to be markedly decreased by 5 days of culture (Fig. 7). In addition, all of the isolated pancreatic epithelium grown on basement membrane gel in the presence of various blocking antibodies still showed evidence of carbonic anhydrase II expression (Figs. 3 and 4), despite the relative absence of formed ducts. The number of carbonic anhydrase II–positive cells in the laminin antibody–treated group was not directly quantified. Precise quantification did not seem to be necessary, because the key finding concerned the numerous non-duct cells that were positively stained, not the relative numbers of positive cells in each culture condition.

The lack of correlation between the morphological formation of pancreatic ducts and the expression of duct-specific markers, particularly in the basement membrane gel, suggests that duct cell differentiation and duct morphogenesis are independent processes. These results also suggest that a component in Matrigel, other than laminin, can maintain carbonic anhydrase II expression in the epithelial cells, despite the absence of ducts. This maintenance of carbonic anhydrase II expression may represent progenitor cells with the potential to form ducts, such as the carbonic anhydrase II–positive cells seen in the freshly harvested E 11.5 pancreas.

There are several possible explanations for the lack of interdependence of duct morphogenesis and duct-specific gene expression. One explanation is that the carbonic anhydrase II–expressing cells represent pancreatic epithelial cells that have the capacity to form ducts, rather than representing only committed duct cells. Another possibility is that the antibodies are cross-reacting with other proteins or isoforms of carbonic anhydrase, which may be present in these embryonic tissues. However, no such cross-reactivity was seen when the antibodies were used to stain adult pancreas or salivary gland (data not shown).

In summary, our data suggest that the cross-region of laminin-1, in addition to the α 6 -containing integrin receptor, is necessary for the induction of pancreatic ductal morphogenesis. Absence of laminin-1 contact may lead to endocrine differentiation from pancreatic ductal/epithelial pluripotent cells. The spatiotemporal pattern of laminin-1 expression in the embryonic pancreas is entirely consistent with this conclusion.
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REFERENCES

43. Wiesen JF, Young P, Web Z, Cunha GR: Signaling through the stomal epit.
dermal growth factor receptor is necessary for mammary ductal development. Development 126:335–344, 1998


