Duct-like Morphogenesis of Longnecker Pancreatic Acinar Carcinoma Cells Maintained in Vitro on Seminiferous Tubular Basement Membranes


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ABSTRACT

We have investigated the behavior of dissociated cells of a moderately differentiated Longnecker transplantable pancreatic acinar carcinoma (Longnecker et al., Cancer Lett., 7: 197–202, 1979) maintained in vitro on acellular seminiferous tubular basement membranes of rat testis. The tumor cells, which grow as solid masses in vivo with little organization, undergo organogenesis in vitro into distinct duct-like structures with lumina in the presence of basement membrane scaffoldings. These duct-like structures were formed by flattened epithelial cells, which exhibited poorly differentiated acinar cell characteristics with few or no zymogen (secretory) granules. The cells lining the duct-like structures retained the pancreatic acinar cell specific antigen as determined by indirect immunofluorescence. DNA synthesis did not accompany duct-like organization; however, all of the cells lining these structures continued to incorporate [3H]thymidine for up to 4–5 days of culture. They continued to synthesize and secrete amylase, a marker protein of pancreatic acinar cells, into the medium. These results demonstrate that neoplastic epithelial cells of Longnecker pancreatic tumor differentiate into duct-like structures when they come into contact with a basement membrane scaffold and do not accumulate well-formed secretory granules. This is in marked contrast to the previously reported in vitro differentiation of cells derived from another transplantable rat pancreatic acinar cell carcinoma where the neoplastic cells were fully cytodifferentiated on seminiferous tubular basement membrane without forming duct-like structures but accumulated abundant well-developed zymogen granules (Watanabe et al., Cancer Res., 44: 5361–5366, 1984). Although the basal lamina promotes differentiation of cells of two different pancreatic carcinomas in vitro, we conclude that the in vitro expression of morphogenetic and cytodifferentiation patterns is dependent upon the intrinsic properties of cells of these two transplantable pancreatic tumors.

INTRODUCTION

ECM4 appears to regulate various developmental processes such as cell migration, proliferation, and differentiation (1–7). Several recent studies have demonstrated that different types of normal epithelial cells grown in and on collagen matrices in vitro exhibit organization into three-dimensional tissue-like structures (7–16). Similarly capillary endothelial cells embedded in collagen gels showed the capacity to form capillary-like networks in vitro (17). Thus it appears that collagen and other ECM components including the basal lamina exert an inductive role on the expression of morphogenetic capabilities in vitro by normal epithelial and endothelial cells similar to that occurring in vivo (3, 5, 7, 12, 17). However, very little information exists regarding the role of basement membrane or ECM in the regulation of growth, differentiation, and morphogenesis of "neoplastic" cells in vitro, despite the notion that failure to maintain a basement membrane may be involved in the neoplastic disorganization of epithelial tissue architecture in vivo (18, 19). The present studies were undertaken to examine the influence of basement membrane scaffolding on in vitro growth and rearrangement of neoplastic acinar cells. We now report that dissociated cells from a moderately differentiated transplantable pancreatic acinar carcinoma (20), maintained in vitro on seminiferous tubular STBMs, organize into distinct duct-like structures, without evidence of abundant secretory granules unlike the previously reported observations of complete cytodifferentiation with well-developed abundant zymogen granules in neoplastic pancreatic acinar cells of another transplantable pancreatic tumor (21), when similarly maintained in vitro on STBM (22). This model system of STBM and its components laminin and type IV collagen may be useful for delineating the mechanisms involved in differential regulation of differentiation and organization of cells.

MATERIALS AND METHODS

Materials purchased were as follows: L-[3,4,5-3H]Leucine (146 Ci/mmol) from Amersham Corp., Arlington Heights, IL; [methyl-3H]thymidine (1 Ci/mmol) from Research Products International Corp., Mt. Prospect, IL; DNase 1, soybean trypsin inhibitor, deoxycholate, and bovine serum albumin (fraction V) from Sigma Chemical Co., St. Louis, MO; collagenase (class IV) from Worthington, Freehold, NJ; SDS, acrylamide, and related gel electrophoresis reagents from Bio-Rad, Richmond, CA; and amphoterpine (pH 3.5–10) from LKB Instruments, Inc., Gaithersburg, MD. CDM was prepared as described by Parse and Marsh (23); this was supplemented with vitamin C (10 mg/liter), hydrocortisone (4 mg/liter), insulin (8 mg/liter), and STI (0.2 mg/liter). The CDM also contained the antibiotics gentamicin (50 mg/liter), penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml). Other chemicals were standard reagent grade.

Preparation of Rat Testicular Seminiferous Tubular Basement Membrane. Acellular STBMs were prepared from adult F344 rats by a modification of the procedure (22) outlined by Meezan et al. (24) for the isolation of basement membranes from blood vessels and renal glomeruli. Testes were removed surgically under sterile conditions. After the tunica was dissected out, the loose strands of seminiferous tubules were teased apart in water containing 0.05% sodium azide. The blood vessels and...
interstitial capillary network were removed. Seminiferous tubules were chopped into ~5 mm segments with a razor blade. After these tubules were washed in fresh sodium azide solution for 45 min at room temperature, the loose elements of germinal epithelium were removed by applying gentle mechanical pressure with a rubber policeman; the seminiferous tubules were then washed out and treated with DNase I (100 Kunitz units/ml) for 45 min at room temperature. Finally the seminiferous tubules were treated with 1% sodium deoxycholate for 45 min at room temperature. The germinal epithelium-denuded acellular STBMs (Fig. 1A) were collected and washed at least three times with 0.9% NaCl solution containing 0.2% bovine serum albumin and once with CDM.

**Dissociation of Pancreatic Acinar Carcinoma Cells and In Vitro Maintenance.** Azaserine-induced pancreatic acinar carcinoma (CP-33378) maintained as a transplantable tumor in Wistar/Lewis rats (20) was dissociated into single cells according to the procedures described previously (25-27). Tumor cells (1 × 10^6 to 1 × 10^7 in 0.1 ml Krebs-Ringer bicarbonate buffer) were mixed with supplemented CDM containing ~50-75 STBMs. The culture dishes were then placed on a Belco rocker platform in an incubator at 37°C in an atmosphere of 5% CO₂ and rocked gently for 4 to 6 h to facilitate contact and attachment of tumor cells to the floating STBMs. The unattached cells were removed within 12 h, and tumor cell-STBM aggregates maintained in culture for 7-10 days.

**Tissue Autoradiography.** Incorporation of [³H]leucine (20 μCi/ml) into proteins and [³H]thymidine (120 μCi/ml) into DNA by tumor cells maintained on STBMs was assessed by light and electron microscopic autoradiography as described previously (28). The cells on STBMs were labeled either continuously for up to 96 h or for 1 h prior to harvesting at 48, 72, and 96 h.

**Morphological Procedures.** Unfixed STBM-tumor cell aggregates were photographed using a Zeiss Ultraphot III microscope using either phase optics or with lowered condenser. For light microscopy, the STBM-tumor cell complexes and fragments of transplantable tumor were fixed in Bouin’s fluid and embedded in paraffin, and 3-4-μm-thick sections were stained with hematoxylin and eosin. For transmission electron microscopy, samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h, postfixed in 1% OsO₄, and processed as described previously (28).

**Immunofluorescence.** For fluorescence microscopy, pancreatic acinar carcinoma cells maintained in vitro on STBM for 3 to 7 days, as well as portions of transplanted tumor, were fixed in Bouin’s fluid and embedded in paraffin. Sections 3-4 μm thick were cut and processed for the indirect immunofluorescence localization of pancreatic acinar cell specific antigen using the monoclonal rat anti-mouse pancreatic acinar cell (ACR-1) antibodies as described previously (29).

**[^35S]Methionine Labeling.** Pancreatic acinar carcinoma cells were maintained on STBM for 4, 24, 48, and 72 h. They were labeled with[^35S]methionine (100 μCi/ml methionine-free medium) for 4 h before harvesting at the time intervals indicated. At the end of the designated incubation period, the STBM cell aggregates were pelleted (2 × 500 rpm/10 min). The lysates of the cells were prepared and processed for one-dimensional electrophoresis as indicated below. Similarly the medium was centrifuged at 50,000 rpm for 45 min to sediment cell debris and the supernatant was analyzed.

**SDS-Polyacrylamide Gel Electrophoresis.** One-dimensional electrophoresis of[^35S]methionine-labeled tumor cell lysates and the culture medium was performed on SDS-polyacrylamide slab gels according to the method of Laemmli (30). The gels were dried and autoradiograms were prepared.

**Immunoblot Analysis.** The secretory proteins in the incubation medium of tumor cells maintained on STBM for 48-72 h were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis and were transferred electrophoretically to nitrocellulose sheets. The sheets were treated with rabbit anti-rat amylase antiserum and then [³H]-labeled protein A and autoradiographed.

**RESULTS**

**Morphological Studies.** Azaserine-induced Longnecker transplantable pancreatic acinar carcinoma of the Wistar/Lewis rat was dissociable into viable single cells by the method used for the dissociation of nafenopin-induced pancreatic acinar carcinoma of the Fischer 344 rat established by Reddy and Rao (21). Dissociated tumor cells attached readily to the STBM within 1 to 4 h (Fig. 1B). This adhesive property enabled the removal of excess or nonadherent tumor cells from the culture medium. The cell density on STBM was adjustable either by the number of tumor cells added in relation to the quantity of STBM segments in culture dishes or by varying the length of contact time with STBM prior to removal of free cells from the culture medium. The tumor cell-STBM aggregates floated freely in the medium as individual segments or formed entangled aggregates. These, however, did not attach to the plastic surface.

The general morphological pattern of these tumor cell-STBM aggregates was monitored with an Ultraphot III microscope. Within 8 to 12 h, the tumor cells attached to STBM began to flatten out somewhat and appeared to establish cell to cell contact. By 18 to 24 h, small gland-like structures with refractile cavities developed. Within 24 to 96 h, the entire surface of the STBM segments was covered with numerous duct-like structures (Fig. 1C). The size of these duct formations increased progressively with time for up to 5 days. By phase contrast microscopy, their lumina appeared to contain translucent fluid (Fig. 1C). These cavities exceeded trypsin blue, suggesting that they were in fact authenticated lumina surrounded by tumor cells and that they do not represent the lumina or "holes" of the STBM lined by the cells.

The histological appearance of the Longnecker pancreatic acinar carcinoma maintained in vivo as transplanted tumor and of duct-like formations by tumor cells maintained in vitro on STBM are shown in Fig. 2, A and B, respectively. Under in vivo conditions, this less-well-differentiated tumor displayed the appearance of a solid mass of relatively small cells, without evidence of gland formation (Fig. 2A). By electron microscopy, the cells of this tumor showed usual arrays of rough endoplasmic reticulum and a few variable numbers of zymogen (secretory) granules in some tumor cells (not illustrated). In contrast, the histology of dissociated cells of this tumor maintained in vitro on STBMs showed duct-like morphogenetic organizations (Fig. 2B). Duct-like structures consisted of a lumen surrounded by flattened epithelial cells resting on the STBM.

Examination of semithin sections of tumor cell-STBM aggregates demonstrated that the majority of pancreatic acinar cells delimited a lumen. Thin sections showed that the duct-like structures consisted of three or more cells that resembled acinar cells with their bases resting on the STBM (Fig. 3) and their apices with few microvilli toward the lumen (Fig. 4A). The lateral plasma membranes of these cells had points of apposition resembling tight junction formation (Fig. 4B). The cells contained large nuclei and prominent strands of rough endoplasmic reticulum. The zymogen granules were sparse in the majority of cells lining these duct-like structures. When present, these granules were irregular in shape and smaller than typical zymogen granules (Figs. 3A and 4).

Pancreatic carcinoma cells plated in plastic dishes in the absence of STBMs formed solid clumps or sheets in the culture
medium and displayed no gland-like organizations. Deletion of soybean trypsin inhibitor from the culture medium did not inhibit gland-like formations in cells maintained on STBM.

**Immunofluorescence Studies.** In order to determine whether duct-like formations in vitro result in the persistence or loss of gland-like formations in cells maintained on STBM.

**DNA and Protein Synthesis.** DNA synthesis did not accompany the reorganization of pancreatic acinar cells on STBM into duct-like structures. Although 10–15% of the freshly dissociated neoplastic cells incorporated [3H]thymidine, none of the cells maintained on STBM for up to 4 days in the presence of [3H]thymidine showed any nuclear labeling by electron microscopic autoradiography (not illustrated). However, all these cells continued to incorporate [3H]leucine as evidenced by autoradiography indicating their viability (Fig. 3B).

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Studies.** Autoradiograms of one-dimensional gel electrophoresis of lysates of the neoplastic cells and media showed the consistent presence of an amylase band (M, ~55,000) during the entire period of labeling (Fig. 5). The identity of the band as amylase was confirmed by the immunoblot analysis where a single band corresponding to the M, ~55,000 was seen (Fig. 5).

**DISCUSSION**

The present study demonstrates that dissociated neoplastic pancreatic cells derived from azaserine-induced transplantable acinar carcinoma (20), when maintained on STBM, undergo organization into duct-like structures. This peculiar morphogenetic change, perhaps, represents one of the solitary examples where basal lamina as a whole or one of its constituents is capable of influencing the dedifferentiated neoplastic epithelial cells in vitro into the formation of organized ductules, structures which are very much reminiscent of the histological architecture of normal pancreas. On the other hand, in vivo these neoplastic cells lack epithelial organization, cell orientation, or gland formation (20). The tumor cells of another transplantable pancreatic acinar carcinoma established in a F344 rat by Reddy and Rao (21) fail to organize in vivo into discernible acinar patterns but exhibit polarization of secretory granules especially in perivascular areas (31). Thus it appears that STBM play a significant regulatory role in the inductive assemblage of singly dispersed cells into ductules. Conversely it can be safely stated that the inability to produce or maintain complete and structural basal lamina in vivo may be responsible for the failure of neoplastic cells to undergo morphogenetic or histogenetic differentiation. Indeed Ingber et al. (18) reported the absence or abnormally scanty distribution of certain components of basal lamina, i.e., type IV collagen and laminin, in another transplantable pancreatic tumor established in F344 rats by Reddy and Rao (21). These observations do suggest a role of basal lamina, whether derived from STBM or other sources, in influencing the morphogenesis of neoplastic cells.

Available evidence strongly supports the role of mesenchyme and ECM components including basement membrane as informative or inductive molecules in growth regulation and morphogenetic organization of normal epithelial tissues under in vitro and in vivo conditions (2–6, 32–35). Several examples of the inductive effect of collagen overlays and matrices on the establishment of tissue-like structures by normal epithelial cells in culture have been reported. These include cells from mammary gland (8, 11, 16), submandibular gland (36), and thyroid (9), as well as established cell lines derived from canine kidney (12) and endothelial cells of bovine adrenal cortex (17). In most of these investigations, the behavior of either normal cells or cultured cell lines was studied. Our results provide, for the first time, an unequivocal example of the in vitro reorganization of neoplastic pancreatic acinar cells in the presence of a basement membrane scaffolding, although these cells are derived from a solid tumor with disorganized architecture and maintained in vivo for several transplant generations. However, they retain the capacity to form distinct morphogenetic patterns in vitro if they are provided with appropriate inductive environment. The striking difference in the cytodifferentiation potential between F344 rat pancreatic acinar carcinoma cells and Longnecker pancreatic acinar carcinoma cells when maintained in vitro on STBM suggests that basal lamina or some component of basal lamina is sufficient to exert differentiative stimulus, but we think that the ability to express morphogenetic patterns and to accumulate secretory granules is due to intrinsic differences between these two pancreatic acinar tumors. It appears that Longnecker pancreatic acinar carcinoma cells rapidly discharge secretory proteins into the medium and possess very little intrinsic capability for storage.

The interaction of pancreatic carcinoma cells with the STBM appears sufficient to induce the characteristic duct-like histological architecture in the absence of DNA synthesis and cell replication. A specific mesenchymal factor (33, 34) appears to control
cell proliferation and cytodi↵erentiation during embryonic develop-
ment and may be required for the proliferation of these cells in vitro. The present studies, however, suggest that this factor may not be essential for epithelial polarization and cell-cell inter-
action. The structures formed under the in vitro experimental con-
dition described in the present study depict the histological appearance of ductular structures in normal rat pancreas and the pancreatic pseudoductules induced under certain experimental condi-
tions (37–41). However, the duct-like structures lining the vacuoles described in the present study retain acinar cell-speci↵ic antigen and secrete amylase in spite of the absence of charac-
teristic cytodi↵erentiation features of acinar cells. This is par-
icularly relevant in relation to our earlier study in which neoplastic pancreatic acinar cells derived from another transplantable tumor accumulated numerous secretory granules and exhibited char-
aracteristic acinar cytodi↵erentiation when maintained in vitro on
STBM (22). However, those cells failed to undergo histogenetic or morphogenetic di↵erentiation such as the one observed in the presen
study. It would be of particular interest to ascertain whether the cells lining the duct-like structures formed under in vitro conditions can be induced to express alkaline phosphatase and carbonic anhydrase similar to that observed in the 5-bromo-
2-deoxyuridine-treated embryonic pancreas (42).

The mechanism by which collagen and other ECM components induce organogenesis and morphogenesis is not well under-
stood. The formation of polarized epithelium, cell-cell interaction, and tissue-speci↵ic architectural imprint may be intrinsic prop-
erties of normal and in certain instances of neoplasia cells as well, but these may be inluenced by specific components of the
basement membranes. Among the basement membrane compo-
nents which could modulate the cytodi↵erentiation events are
various species of sul↵ated and nonsul↵ated proteoglycans. The
nonsul↵ated proteoglycans which have been shown to inluence
the morphogenesis include hyaluronic acid (2, 4) while the sul-
fatized one is chondroitin sulfate (7, 43). In addition, the heparan
sulfate-proteoglycan has been implicated in the regulation of
embryonic-morphogenetic developmental events (44, 45). Ad-
mittedly the role of proteoglycans in this investigation is not
proven but would be one of the important issues which needs
to be addressed in future investigations. Finally it seems that
our relatively simple model system will greatly facilitate the invest-
gation of the events involved in the morphogenesis and reorgan-
ization of neoplastic epithelial cells.

REFERENCES

DUCT-LIKE MORPHOGENESIS OF A PANCREATIC CARCINOMA


Fig. 1. Light microscopic appearance of isolated acellular STBM segment of rat testis (A) and pancreatic acinar carcinoma cells maintained in vitro on such STBM for 4 h (B) and 48 h (C). The STBM in A, stained with 0.01% toluidine blue, shows the hollow tubular configuration with arrows pointing to the lumen. In B, pancreatic acinar carcinoma cells attached to the STBM (bm) at 4 h of culture show marked tendency to aggregate into clumps of several cells. In C, pancreatic carcinoma cells maintained on STBM for 48 h reveal duct-like structures with refractile lumina (d). A few zymogen granules (arrows) are seen in some cells. A, x 40; B, x 400; C, x 1,200.
Fig. 2. Histological (A and B) and immunofluorescent (C and D) micrographs of Bouin's-fixed and paraffin-embedded Longnecker pancreatic acinar carcinoma maintained in vivo as a transplantable tumor (A and C) and pancreatic carcinoma cells maintained in vitro on STBM for 72 h (B and D). Sections in C and D are stained for pancreatic acinar cell specific ACR-1 antigen with monoclonal antibodies. The tumor cells cultured on STBM (bm) reveal duct-like organizations with lumina (d). S, stroma. A, H & E, × 180; B, H & E, × 400; C, × 540; D, × 220.
Fig. 3. Low power electron micrographs of pancreatic acinar tumor cells cultured on STBM for 72 h (A and B). Pancreatic tumor cells are organized around circular to oval lumina (LUMEN) to form duct-like structures. These structures are surrounded by STBM (bm). A few cells lining these duct-like structures contain focal collections of small secretory granules in the cytoplasm facing the luminal surface. B, autoradiograms showing [3H]leucine incorporation in cells lining the duct-like structures. N, nucleus; Er, endoplasmic reticulum. A, × 1,900; B, × 2,800.
Fig. 4. Electron micrographs of ductules indicating the detailed structure of the cells. The cells contain plentiful rough endoplasmic reticulum and some zymogen granules (gr). The junctions are readily seen between the adjoining cells (arrows). B, higher magnification of the inset of A to better illustrate the presence of granules (gr) and cell junctions (arrows). A, x 8,000; B, x 30,000.
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