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Establishment of three-dimensional cultures of human pancreatic duct epithelial cells

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Abstract

Three-dimensional (3D) cultures of epithelial cells offer singular advantages for studies of morphogenesis or the role of cancer genes in oncogenesis. In this study, as part of establishing a 3D culture system of pancreatic duct epithelial cells, we compared human pancreatic duct epithelial cells (HPDE-E6E7) with pancreatic cancer cell lines. Our results show, that in contrast to cancer cells, HPDE-E6E7 organized into spheroids with what appeared to be apical and basal membranes and a luminal space. Immunostaining experiments indicated that protein kinase Akt was phosphorylated (Ser473) and CTMP, a negative Akt regulator, was expressed in both HPDE-E6E7 and cancer cells. However, a nuclear pool of CTMP was detectable in HPDE-E6E7 cells that showed a dynamic concentrated expression pattern, a feature that further distinguished HPDE-E6E7 cells from cancer cells. Collectively, these data suggest that 3D cultures of HPDE-E6E7 cells are useful for investigating signaling and morphological abnormalities in pancreatic cancer cells.

Keywords

pancreatic ductal epithelial cells; pancreatic cancer; Akt; CTMP; three-dimensional culture; signal transduction; nuclear localization; MCF-10A

Introduction

Dynamic reciprocal interactions between cells and the extracellular matrix generate biochemical signals that regulate normal embryonic morphogenesis and tissue homeostasis in the adult organism [1,2]. During carcinogenesis, however, histological features essential for epithelial cell architecture and function, such as a polarized morphology, specialized cell-cell contacts, and basement membrane interactions, are disrupted. Thus a model system in which such cell–extracellular matrix interactions take place may reveal differences among normal, non-malignant cells, and cancer cells that can be used to help model the early stages of carcinogenesis during which epithelial cell architecture is disrupted [1,2]. Evidence of the

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promise of this approach has already been observed for mammary epithelial cells, which when grown in three-dimensional (3D) cultures, but not in monocultures, were observed to form polarized spheroids that recapitulate many aspects of glandular architecture as those seen *in vivo*, thereby yielding invaluable information about breast epithelial morphogenesis and neoplasia.

Indeed, breast epithelial cells grown in a 3D environment form structured acini and hollow lumens and also secrete milk proteins [1-3], in stark contrast to tumorigenic breast cell lines, which form highly disorganized structures [4]. Further, the fact that the normal phenotype of these cells was restored by blocking the β 1-integrin receptor or by inhibitors of the phosphoinositide 3-kinase (PI3K) signaling pathway showed that this tendency of tumorigenic cells to form misshapen aggregates in 3D cultures was due to aberrant signaling [5,6].

To establish such a model system for the study of pancreatic ductal cancer, one of the most malignant and poorly understood human cancers, we utilized recently established and characterized human pancreatic duct epithelial cells (HPDE-E6E7) [7]. HPDE-E6E7 cells have several characteristics that make them well suited to this application. In particular, they have limited genetic aberrations, grow in an anchorage-dependent manner, and are non-tumorigenic in mice [8]. Further, stable expression of oncogenic Ki-Ras^{G12V} caused transformation of HPDE-E6E7 cells and activated signaling molecules implicated in human pancreatic cancer development such as the PI3K protein kinase mediator Akt [9].

Our results indicate that HPDE-E6E7 cells form highly organized structures in 3D cultures. In addition, HPDE-E6E7 cells could be distinguished from cancer cells on the basis of the nuclear expression pattern of a negative regulator of Akt known as the carboxyl terminal modulator protein (CTMP) [10].

Materials and Methods

Cell lines and reagents

Panc-1, MIA PaCa-2, Su.86.86, and BxPC-3 pancreatic cancer cells purchased from the American Type Culture Collection (ATCC, Manassas, VA) were maintained in RPMI or Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS) and cultured according to ATCC protocols. MCF-10A cells were gifted by Dr. Yiling Lu (M. D. Anderson Cancer Center) and cultured as described elsewhere [2]. HPDE-E6E7 (HPDE-E6E7) pancreatic duct epithelial cells, which were a generous gift from Dr. Ming-Sound Tsao (Ontario Cancer Institute, Toronto, Ont., Canada), were cultured in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (Invitrogen, Carlsbad, CA) as described elsewhere [7].

The following antibodies were purchased: CTMP, phosphoAkt S473, and Akt1 (Cell Signaling Technology, Danvers, MA); Akt1/2 and phosphoERK (Santa Cruz Biotechnology, Santa Cruz, CA); pan ERK (BD Transduction Laboratories, San Diego, CA); and tubulin (Sigma, St. Louis, MO). Secondary western blotting antibodies, Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) and Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L), were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) Alexa Fluor® 546 goat anti-rabbit IgG (H+L), Alexa Fluor® 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L), 4'-6'-diamidino-2-phenyl-indole, dihydrochloride (DAPI), MitoTracker® mitochondrion-selective probe, and Calcein AM fluorogenic esterase substrate were obtained from Invitrogen Molecular Probes.

Overlay 3D culture

MCF-10A, HPDE-E6E7, and various pancreatic cancer cell lines were cultured on matrigel as described elsewhere [11]. Briefly, 40 μ l of cold (4°C) growth factor-reduced Matrigel (Becton

Dickinson, Franklin Lakes, NJ) was added to each well of an eight-well glass chamber slide (Becton Dickinson) and spread evenly using a pipettor tip. While the Matrigel solidified at 37°C, a plate of confluent cells was harvested with trypsin, after which cells were centrifuged at 100 × g in a tissue culture centrifuge for 5 min. Pelleted cells were then gently resuspended to obtain a homogeneous suspension in medium lacking EGF or FBS. MCF-10A cells (25,000 cells/ml), pancreatic cancer cell lines (10,000 cells/ml), or HPDEE6E7 cells (30,000 cells/ml) were mixed (1:1) with a solution of 4% cold Matrigel containing either 10 ng/ml EGF or 10% FBS, as needed. Then, 400 µl of the suspension was overlaid on top of the solidified Matrigel. Cells were grown in a 5% CO₂ humidified incubator at 37°C for the indicated days, with the medium replaced on the 4th day with fresh medium containing 2% Matrigel and 5 ng/ml EGF or 5% FBS.

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were cultured as described for the overlay 3D culture. Medium was removed from each well of the chamber slide, and cells were immediately fixed with 500 µl of freshly prepared 2% paraformaldehyde (pH 7.4) for 10–12 min. Cells were then permeabilized with 500 µl of TBS (25 mM Tris base, pH 7.4, 136 mM NaCl, 2.7 mM KCl) containing 0.5 % Triton X-100 for 10 min at room temperature. Each well was rinsed three times with 500 µl of TBS containing 0.05 % Tween-20 (TBST) for 10–15 min at room temperature, following which permeabilized cells were incubated with 200 µl/well TBS and fatty acid-free bovine serum albumin (3%) for 1 hr at room temperature. Following the blocking step, cells were incubated with primary antibody (1:100) in 100 µl of block solution overnight (15–18 hr) at room temperature. Each well was rinsed three times (20 min each) with TBST at room temperature with gentle rocking, after which cells were incubated with fluorescent conjugated secondary antibody (1:200) containing 100 µl of blocking buffer for 1 hr at room temperature. After three rinses with TBST, cells were incubated with DAPI (30 min) at room temperature and then rinsed again three times with TBS for 10 min each time. Finally, 200 µl of freshly prepared Prolong Antifade Reagent (Molecular Probes, Invitrogen) was added to each well overnight. The chamber slides were stored at 4°C in a moist chamber before being examined under a Nikon Eclipse TE2000S or an Olympus IX70 Inverted immunofluorescence microscope. Calcein AM, which is cleaved into its fluorescent form by active esterases, was used to stain intact cell membranes. MitoTracker®, which fluoresces after it is accumulated and reduced, was used to stain active mitochondria.

Western Blotting of Spheroid proteins

Matrigel cultures were rinsed with cold PBS and then trypsinized for 20–30 min. Cells were collected by centrifugation (100 × g) and incubated in 100 µl of hypotonic lysis buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml leupeptin, 0.5 µg/ml benzamidin, and 2 µg/ml aprotinin) on ice. After 30 min, 10% NP-40 was added (final concentration: 0.3 %), and the solution was vigorously vortexed before centrifugation at 10,000 × g for 2 min to pellet nuclei. The supernatant, which was expected to contain a crude mixture of cytosolic and microsomal protein fractions, was removed. The nuclear pellet was incubated on ice with 15 µl of extraction buffer (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/ml leupeptin, 0.5 µg/ml benzamidin, and 2 µg/ml aprotinin) and frequently vortexed for 30 min. Cell debris was removed by centrifugation at 10,000 × g for 5 min to obtain the crude nuclear fraction. Protein concentration was determined using Bradford reagent (Biorad). Proteins were resolved on a 12% poly-acrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted for phosphorylated Akt (S473), total Akt, total cJun, and CTMP. Bands were visualized by ECL detection reagents (Amersham, Piscataway, NJ).

Results and Discussion

Human pancreatic duct epithelial cells form organized structures on matrigel

To establish a system for pancreatic cancer cells similar to the one established for MCF-10A mammary epithelial cells [1,2], we cultured HPDE-E6E7 cells in Matrigel using a modified protocol previously established for the MCF-10A cells [11]. Remarkably, similar to the MDF-10A cells, HPDE-E6E7 cells also organized themselves and grew into large spheroids (Fig. 1A). Also similar to the MDF-10A cells, light microscopic examination of the HPDE cultures revealed that their spherical structures likewise began to form a hollow perceptible lumen around day 8 (Fig. 1B). Thus, HPDE-E6E7 cells did not form simple aggregates of cells but instead formed specialized structures that appeared to have a monolayer of polarized cells around a cavity. Interestingly, however, while MCF-10A cells developed spheroids that were largely isolated and distinct, HPDE-E6E7 cells formed two types of structures: isolated spheroids and spheroids that were joined to each other by bridge-like connections (Fig. 1A). Whether the two types of spheroids are truly different from each other and formed by different subpopulations of pancreatic ductal cells is unclear and requires further investigation.

To determine if pancreatic cancer cells would also organize into such spheroids, we evaluated the growth of various cell lines (MIA PaCa-2, Panc-1, BxPC-3, and SU.86.86) in Matrigel using two different serum concentrations. Unlike the HPDE-E6E7 cells, BxPC-3, Panc-1, and to a great extent, MIA PaCa-2 cells formed disorganized colonies (Fig. 1C). The SU.86.86 pancreatic cancer cells were an exception, in that they also appeared to form spheroids, though the size of the spheroids depended on the serum concentration and the spheroids were significantly larger than those formed by HPDE-E6E7 cells. It is not clear why SU.86.86 cells become arranged into spheroids and MIA PaCa-2, Panc-1, and BxPC-3 cells do not and if the SU.86.86 spheroids are identical in their organization to those generated by HPDE-E6E7 cells. In contrast to 3D cultures, monolayers do not reveal such phenotypic differences between pancreatic normal and tumor cells. That some pancreatic cancer cells form spheroids was also shown in a study of Capan-1 cells that used a Brunswick agitator to generate 3D cultures as a model system for the investigation of ion-exchange processes in the human pancreas [12]. The spheroids in this case were hollow, polarized spherical structures that were bound by tight junctions and desmosomes. Similar studies of 3D cultures of other pancreatic cancer cell lines revealed novel aspects regarding DNA repair and the recombination factor Rad51 [13] and showed that epimorphin was involved in spheroid development [14].

The inability of many pancreatic cancer cell lines to form well-organized spheroids is consistent with what has been reported for breast tumor cells [15]. However, the molecular basis for the organization of cells into spherical structures is poorly understood, though β 1-integrin may play a role [5]. Indeed, blocking β 1-integrin with an antibody caused malignant HMT-3522 T4-2 cells to revert to the normal HMT-3522 S1 phenotype when grown under 3D conditions [5]. Follow up studies showed that the reversion was observed only in 3D cultures and not when the same cells were grown and treated as monolayers [15].

We next employed the fluorescent dyes Calcein AM and MitoTracker® to determine if cells were actively dividing and growing and if any cell death had occurred during the formation of the 3D structures. As seen in Fig. 1D, HPDE-E6E7 spheroids possessed what again appeared to be apical and basal membranes with a center, whereas the pancreatic cancer cell lines seemed to have lost their ability to organize themselves into similar structures. Also as seen previously, although both HPDE-E6E7 and SU.86.86 cells formed spheroids, SU.86.86 cells did not possess the characteristic lumen and apical and basal membranes. The fluorescent dyes further indicated that the cells were actively metabolizing and proliferating within the 3D structures of both HPDE-E6E7 and pancreatic cancer cells.

Akt activation and CTMP localization in HPDE-E6E7 and pancreatic cancer cells

Previous studies in MCF-10A cells have shown that the PI3K/Akt pathway is a crucial regulator of spheroid formation and maintenance [6,16]. Aberrant activation of Akt led to the disrupted formation and loss of organization of MCF-10A spheroids in an mTOR-dependent manner [16]. There is increasing evidence that the PI3K/Akt pathway is also dysregulated in pancreatic cancer and is essential for the survival and proliferation of pancreatic cancer cells [17]. Persistent activation of the pathway in this cancer may be due to various factors, including oncogenic Ki-Ras [18], growth factor receptor-ligand systems [19], Akt2 amplification [20], and epigenetic silencing of the PI3K antagonist Pten [21]. In addition to the Pten-mediated dephosphorylation of phosphoinositides, Akt activity is also negatively regulated when Akt binds to proteins such as CTMP [10].

As an initial approach to investigating this pathway in 3D cultures, we examined Akt phosphorylation. Immunostaining of day 2-8 spheroids revealed that Akt was phosphorylated on Ser473 (Fig. 2) and that it was present mostly in the cytoplasm of HPDE-E6E7 cells. It has been shown that the Akt-binding protein CTMP is mostly present in the cytoplasmic and membrane fractions of cells and that it colocalizes with Akt at the plasma membrane [10]. In the present study, our results suggest that CTMP is expressed throughout the cell and that it is present even in the nucleus (Fig. 2). To our surprise, however, nuclear CTMP in HPDE-E6E7 cells became highly concentrated by day 4 and no longer appeared to colocalize with Akt to the same extent as it did on day 2. In contrast to CTMP, phospho-Akt appeared to localize in the cytoplasm and outer membranes of the cells and its nuclear expression pattern did not change. Western blotting analysis confirmed that CTMP was in the nucleus but suggested that its dynamic nuclear expression pattern was due to a redistribution of nuclear CTMP rather than any influx from its cytoplasmic pool (Fig. 3).

We next investigated if there was a difference in CTMP localization between HPDE-E6E7 and pancreatic cancer cell lines. Interestingly, the concentrated nuclear CTMP expression pattern was observed much earlier (day 2) in the 3D pancreatic cancer cell structures than in HPDE-E6E7 spheroids (Fig. 4). The nuclear CTMP pattern that was strikingly bright and concentrated on day 4 appeared to become less pronounced by day 8 especially in HPDE-E6E7 spheroids.

Together, our data suggest that HPDE-E6E7 3D cultures may constitute an important system for modeling the early stages of pancreatic carcinogenesis. These data also revealed a novel localization pattern for CTMP and raised the possibility that CTMP function and/or regulation may be different in HPDE-E6E7 and pancreatic cancer cells. Although the function of nuclear CTMP is unknown, it is conceivable that it would regulate Akt's nuclear functions [23]. It is also possible that CTMP is associated with specific subnuclear bodies [24] and plays a role that has not yet been identified. Further studies are required to identify the exact location and function of CTMP in the nuclear compartment.

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Abbreviations

3D, three dimensional; HPDE-E6E7, human pancreatic duct epithelial cells; CTMP, C-terminal modulator protein; PI 3-kinase, phosphoinositide 3-kinase.

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Fig 1. HPDE-E6E7 cells form spherical structures on matrigel

A) MCF-10A cells (5000 cells/well) and HPDE-E6E7 cells (6000 cells/well) were overlaid on Matrigel. Cells were observed 1, 4, and 8 days later under a Nikon Eclipse TE2000S Inverted microscope ($\times 10$) and photographed with a Hamamatsu digital camera.

B) HPDE-E6E7 cells (6000 cells/well) were overlaid on Matrigel in 5 ng/ml EGF and 2% Matrigel. Cells were observed 2, 4, and 8 days later under a Nikon Eclipse TE2000S Inverted microscope ($\times 10$) and photographed with a Hamamatsu digital camera.

C) BxPC-3, MIA PaCa-2, Panc-1, and SU86.86 human pancreatic cancer cells were overlaid on Matrigel and photographed ($\times 20$) on days 2, 4, and 8. Human pancreatic cancer cell lines were grown in either 1% or 5% fetal bovine serum to determine the effects of serum factors.

D) HPDE-E6E7 cells and pancreatic cancer cells were stained for active mitochondria using a specific dye (MitoTracker®). To assess cell viability, the cells were stained on each indicated day with a cell-permeable dye (Calcein AM) that becomes fluorescent green following hydrolysis catalyzed by intracellular esterases. Cells were photographed using an Olympus IX70 microscope ($\times 20$).

Fig. 2. Akt phosphorylation and CTMP expression in 3D cultures of HPDE-E6E7 cells

A-C) HPDE-E6E7 spheroids were fixed, permeabilized, and co-stained with various combinations of anti-CTMP, anti-pAkt-S473, anti-total Akt, and anti-tubulin antibodies. They were then incubated with either Alexa Fluor® 546 (Red) goat anti-rabbit IgG (H+L) or Alexa Fluor® 488 (Green) F(ab')₂ fragment of goat anti-mouse IgG (H+L) and visualized using an Olympus IX70 microscope (×20). Shown here are representative fields from three independent experiments for day 4 cultures. Day 2 spheroids are shown in insets.

Fig. 3. Western blot analysis of CTMP localization in HPDE-E6E7 cells

HPDE-E6E7 cells were cultured on Matrigel for the indicated days and then harvested using trypsin and collected. Cells were incubated in hypotonic buffer to prepare crude extracts of cytoplasmic and microsomal, and nuclear proteins. Proteins were subjected to SDS-PAGE electrophoresis, transferred to nitrocellulose, and immunoblotted for the indicated proteins.

Fig. 4. CTMP localization in HPDE-E6E7 and pancreatic cancer cell 3D cultures

3D structures were fixed, permeabilized, and stained with anti-CTMP antibodies. The nucleus was stained with DAPI and photographs were obtained on the indicated days with an Olympus IX70 microscope ($\times 20$).