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Thiol-ene hydrogels as desmoplasia-mimetic matrices for modeling pancreatic cancer cell growth, invasion, and drug resistance

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ABSTRACT

The development of pancreatic ductal adenocarcinoma (PDAC) is heavily influenced by local stromal tissues, or desmoplasia. Biomimetic hydrogels capable of mimicking tumor niches are particularly useful for discovering the role of independent matrix cues on cancer cell development. Here, we report a photo-curable and bio-orthogonal thiol-ene (i.e., cross-linked by mutually reactive norbornene and thiol groups via photoinitiation) hydrogel platform for studying the growth, morphogenesis, drug resistance, and cancer stem cell marker expression in PDAC cells cultured in 3D. The hydrogels were prepared from multi-arm poly(ethylene glycol)-norbornene cross-linked with protease-sensitive peptide to permit cell-mediated matrix remodeling. Collagen 1 fibrils were incorporated into the covalent network while cytokines (e.g., EGF and TGF-β1) were supplemented in the culture media for controlling cell fate. We found that the presence of collagen 1 enhanced cell proliferation and Yes-associated protein (YAP) translocation to cell nuclei. Cytokines and collagen 1 synergistically up-regulated MT1-MMP expression and induced cell spreading, suggestive of epithelial-mesenchymal transition (EMT) in the encapsulated cells. Furthermore, PDAC cells cultured in 3D developed chemo-resistance even in the absence of collagen 1 and cytokines. This phenotype is likely a consequence of the enrichment of pancreatic cancer stem cells that expressed high levels of CD24, sonic hedgehog (SHH), and vascular endothelial growth factor (VEGF).

1. Introduction

Pancreatic cancer is the fourth leading cause of all cancer-related deaths in the US with more than 43,000 new cases and 37,000 deaths in 2012 [1]. Pancreatic ductal adenocarcinoma (PDAC) accounts for more than 80% of all pancreatic cancers, which are difficult to diagnose at an early stage [2]. The late diagnosis and poor prognosis in PDAC lead to limited treatment options and extremely low survival rate [3]. Chemotherapies are generally not effective because PDAC patients quickly develop chemo-resistance by mechanisms that remain elusive [4,5]. Increasing evidence has suggested that the clinical complications associated with PDAC cells are contributed by a subset of cancer cell populations, namely pancreatic cancer stem cells (PCSCs) [6]. Sharing many characteristics with normal stem cells, cancer stem cells not only self-renew, but also differentiate into mature cancer cells. In addition, these cancer stem cells are prone to metastasize and to resist drug-induced apoptosis [7–9]. Although only 1–5% of total PDAC cell population is characterized as PCSCs [10], this cell population is enriched after drug treatment. PCSCs are characterized by the expression of surface markers CD24, CD44, CD133, and epithelial specific antigen (ESA) [6]. These cells also express high levels of sonic hedgehog (SHH), which has been associated with the resistance of PDAC cells to drug (e.g., gemcitabine) treatment [6,11].

While most of the *in vitro* PDAC and PCSC studies were conducted in conventional two-dimensional (2D) tissue culture plastics (TCP), increasing evidence has suggested that cells behave in a more pathophysiological relevant manner when cultured in a three-dimensional (3D) niche [12–16]. 3D matrices derived from animal-based products (e.g., Matrigel, collagen gel, etc.) often possess ill-defined compositions and weak mechanical properties. Furthermore, it may be difficult to study the influence of a specific matrix cue on PDAC cell fate using animal models. We hypothesized that a semi-synthetic microenvironment capable of mimicking

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aspects of pancreatic desmoplasia (i.e., malignant stromal tissues containing high amount of collagen 1, myofibroblastic pancreatic stellate cells, and immune cells) could be used to study how changes in matrix compositions affect PDAC cell behaviors. We have previously shown that hydrogels prepared from norbornene-functionalized 4-arm poly(ethylene glycol) (e.g., PEG4NB) and protease-sensitive peptides supported the growth and morphogenesis of PDAC cells (PANC-1) in 3D [13]. We have also shown that the susceptibility of PANC-1 cells to a peptide drug (NYQQN) in 3D culture is matrix stiffness-dependent [14]. Although these studies shed light on the utility of PEG-based hydrogels on PDAC research *in vitro*, we have not evaluated the synergistic influences of various extracellular matrix (ECM) cues presenting in the desmoplasia on the growth, epithelial-mesenchymal transition (EMT), and drug resistance in PDAC cells cultured in 3D.

To bridge this knowledge gap and to reveal the potential enrichment of PCSCs in 3D culture, we systematically investigate the effects of cell culture platforms on PDAC cell fate processes. We used COLO-357 cells in this study as this cell type is highly sensitive to cytokine (e.g., TGF-β1) and chemotherapeutic treatment (e.g., gemcitabine). To study the influence of matrix cues on PDAC cell behaviors, we utilized a modular synthetic hydrogel platform prepared from a light-mediated orthogonal thiol-norbornene photochemistry [13,14,17]. We evaluated the cytocompatibility of this desmoplasia-mimetic hydrogel system on *in situ* encapsulation of PDAC cells. We also examined the synergistic influence of matrix components (e.g., collagen 1 and cytokines) on PDAC cell proliferation and EMT in 3D. Finally, we studied the impact of culture context on chemo-resistance and enrichment of PCSC-like cells in 3D.

2. Materials and methods

2.1. Materials

4-arm poly(ethylene glycol)-amine (20 kDa) was purchased from JenKem Technology USA. Reagents and chemicals for peptide synthesis were acquired from Anaspec or Chempep. Bovine type I collagen was purchased from Amsbio. AlamarBlue reagents were purchased from AbD Serotec. Live/Dead staining kit for mammalian cells and DAPI stain were obtained from Invitrogen. Gemcitabine was purchased from TSZ CHEM. YAP rabbit mAb, E-cadherin rabbit mAb, vimentin rabbit mAb, anti-rabbit IgG, anti-mouse IgG HRP-linked, and Alexa Fluor® 488-labeled anti-mouse IgG F(ab′)$_2$ antibodies were obtained from Cell Signaling Technology. hVEGF ELISA kit was purchased from PeproTech. HPLC grade acetonitrile and water were acquired from Fisher Scientific and VWR International, respectively. All other chemicals were purchased from Sigma–Aldrich unless noted otherwise.

2.2. Macromer, photoinitiator, and peptide syntheses

PEG-tetra-norbornene (PEG4NB) was synthesized according to an established protocol [18]. Briefly, 4-arm PEG-NH_2 was dried in a vacuum oven overnight and dissolved dimethylformamide (DMF). 5-norbornene-2-carboxylic acid (5 eq. of amine group on PEG) was activated by 2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (HBTU, 5.5 eq.) and hydroxybenzotriazole (HOBt, 5.5 eq.) in DMF for 3 min at room temperature. Next, N,N-diisopropylethylamine (DIEA, 6 eq.) was added into the solution and stirred for 5 min. The 4-arm PEG-NH_2 solution was added to the activated norbornene acid solution. All reactions were performed under nitrogen. After 12 h reaction, the product was filtered and precipitated in cold ethyl ether, dried in a vacuum desiccator, and dialyzed against ddH_2O for 3 days. The degree of functionalization (80–90%) was determined using ^{1}H NMR (Avance III 500, Brüker) (Fig. S1). Photoinitiator lithium arylphosphinate (LAP) was synthesized according to a published protocol without modification [19].

MT1-MMP sensitive linker (KCGPLGLYAGCK) was synthesized using Fmoc-Rink-Amide HBMA resin in a microwave-assisted peptide synthesizer (CEM Discover SPS) following standard HOBt/HBTU coupling chemistry. Peptide cleavage was also performed in the microwave peptide synthesizer (38 °C, 20 W, 30 min) using a cleavage cocktail containing 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS) in the presence of 5% (w/v) phenol. Crude peptide was precipitated in cold ethyl ether, dried overnight in a vacuum desiccator, purified using HPLC (PerkinElmer Flexar System), and characterized by mass spectrometry (Agilent Technologies) (Fig. S2). Purified peptides were lyophilized and stored at −20 °C. The concentration of thiol groups on purified cysteine-containing peptides was quantified using Ellman's reagent (Pierce).

2.3. Collagen-FAM conjugation

To prepare fluorescently labeled collagen 1 for visualizing collagen distribution in hydrogels, bovine type 1 collagen was diluted in PBS at a working concentration of 2 mg/mL. 5(6)-Carboxyfluorescein succinimidyl ester (5(6)-FAM-SE, from Anaspec) was dissolved in dimethyl sulfoxide (DMSO) and diluted 100-fold to yield a working concentration of 1 mg/mL pH of the mixture was adjusted to 7.5 using 7.5% (w/v) of sodium bicarbonate aqueous solution. The mixture was protected from light and incubated on ice for 2 h. The product was used without further purification.

2.4. Cell culture and encapsulation

COLO-357 cells were maintained in high glucose Dulbecco's modified eagle medium (DMEM, HyClone) containing 10% of fetal bovine serum (FBS) (Gibco) and penicillin streptomycin (Gibco, 50 U/mL penicillin, 50 U/mL streptomycin). Prior to cell encapsulation, pre-polymer solutions containing PEG4NB, dithiol cross-linker (DTT or MT1-MMP-sensitive peptide), and 1 mM of LAP were prepared according to the hydrogel formulations shown in Table S1. COLO-357 cells were trypsinized and suspended in a prepolymer solution at 2×10^6 cells/mL. 25 μL of pre-polymer solution was placed in a 1 mL disposable syringe with a cut-open tip. The sample was exposed to 365 nm light at 5 mW/cm^2 for 2 min (Fig. 1A–D).

2.5. Characterization of cell-laden hydrogel

Shear moduli of the cell-laden hydrogels were measured on a digital rheometer (Bohlin CVO 100). To prepare flat gels for rheometry analysis, cell-laden hydrogels were prepared between two glass slides separated by 1 mm thick spacers. Circular discs were punched out from the hydrogel slab using a biopsy punch (diameter 8 mm). Shear moduli of the cell-laden hydrogels were measured at 1- and 10-day post-encapsulation. To visualize cell morphology and collagen distribution, cell-laden hydrogels were collected 1- and 10-day post-encapsulation and fixed with 4% paraformaldehyde solution for 20 min at RT, followed by washing and permeabilization with 1 mg/mL sapponin. The hydrogels were incubated with 100 nM rhodamine phalloidin (Cytoskeleton Inc.) solution for 2 h at RT and counter-stained with DAPI. Confocal images of the stained samples were obtained using an Olympus Fluoview FV100 laser scanning microscope. To identify proliferating cells, 10 nM of EdU reagent (Click-IT® EdU staining kit) was added into the culture media 9 days post-encapsulation, followed by incubation at 37 °C for 24 h. The cell-laden gels were collected and rinsed with PBS twice, fixed with 4% paraformaldehyde solution for 20 min, and permeabilized with 0.5% Triton X-100 for 20 min. The gels were washed with PBS twice and immersed in EdU reaction cocktail prepared following manufacturer's instruction. The gels were counter-stained with DAPI for 1 h and the stained gels were imaged by confocal microscopy.

Cell viability in 2D was determined by MTT assay. COLO-357 cells seeded in 96-well plate at 10,000 cells/well were cultured for 1 day, followed by treatment of cells with gemcitabine-containing media (10^{-1} ~ 10^5 nM) for 4 days (media change every 2 days). After the incubation, the old media were removed and each well was rinsed with PBS. Then, 10 μL of 5 mg/mL MTT solution was diluted in 100 μL of culture medium and added to the cells for 4 h. The purple formazan crystals formed in the cells were dissolved by dimethyl sulfoxide, followed by quantifying the absorbance at 540 nm. Cell morphology was observed using Live/Dead staining and confocal imaging. Cell-laden hydrogels were incubated in Live/Dead staining solution for 1 h at room temperature with gentle shaking. Z-stack images (100 μm thick, 10 μm per slice) of at least four random fields were acquired by using the confocal microscope. Initial cell viability was obtained by counting live and dead cells from 12 randomly selected z-stack live/dead images. The live cell number was divided by total cell count to obtain initial cell viability. Cell cluster diameters were measured 10-day post-encapsulation. For non-spherical clusters, the distance along the longest axis in a cluster was measured instead of diameter.

2.6. RNA isolation, reverse transcription PCR, and real-time PCR

For RNA extraction, gels were collected in DNase/RNase-free microtubes, flash frozen with liquid nitrogen, and stored at −80 °C until use. RNA extraction was performed using a combination of guanidinium thiocyanate-phenol-chloroform extraction [20] and NucleoSpin RNA II kit (Clontech). In brief, frozen gels were homogenized in 900 μL of QIAzol (Qiagen) as the samples thaw and incubated at room temperature for 5 min. The samples were filtered through NucleoSpin Filters to clear lysates before the addition of 180 μL of 1-bromo-3-chloropropane (BCP, Sigma). The mixtures were vortexed for 15 s and incubated at room temperature for 3 min before centrifuging at 13800 g for 15 min at 4 °C for aqueous/organic phase separation. Subsequently, the colorless aqueous layers (~600 μL) were transferred to clean DNase/RNase-free microtubes, followed by the addition of equal volume of RNase-free 70% ethanol, and vortexed for 20 s. The mixtures were then added to NucleoSpin RNA columns, and RNA isolation was performed by following the manufacturer's protocol. The final total RNA products were eluted in 30 μL of DNase/RNase-free water, and the yield was quantified by UV spectrometry. Aliquots of RNA samples were stored at −80 °C until use. In a reverse