Published in final edited form as:

Acta Biomater. 2018 February ; 67: 331–340. doi:10.1016/j.actbio.2017.11.037.

Viscoelastic properties of human pancreatic tumors and *in vitro* constructs to mimic mechanical properties

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is almost universally fatal, in large part due to a protective fibrotic barrier generated by tumor-associated stromal (TAS) cells. This barrier is thought to promote cancer cell survival and confounds attempts to develop effective therapies. We present a 3D *in vitro* system that replicates the mechanical properties of the PDAC microenvironment, representing an invaluable tool for understanding the biology of the disease. Mesoscale indentation quantified viscoelastic metrics of resected malignant tumors, inflamed chronic pancreatitis regions, and histologically normal tissue. Both pancreatitis (2.15 ± 0.41 kPa, Mean $\pm$ SD) and tumors (5.46 ± 3.18 kPa) exhibit higher Steady-State Modulus (SSM) than normal tissue (1.06 ± 0.25 kPa; $p < 0.005$). The average viscosity of pancreatitis samples (63.2 ± 26.7 kPa·s) is significantly lower than that of both normal tissue (252 ± 134 kPa·s) and tumors (349 ± 222 kPa·s; $p < 0.005$). To mimic this remodeling behavior, PDAC and TAS cells were isolated from human PDAC tumors. Conditioned media from PDAC cells was used to culture TAS-embedded collagen hydrogels. After 7 days, TAS-embedded gels in control medium reached SSM (1.45 ± 0.12 kPa) near normal pancreas, while gels maintained with conditioned medium achieved higher SSM (3.38 ± 0.146 kPa) consistent with tumors. Taken together, we have demonstrated an *in vitro* system that recapitulates *in vivo* stiffening of PDAC tumors. In addition, our quantification of viscoelastic properties suggests that elastography algorithms incorporating viscosity may be able to more accurately distinguish between pancreatic cancer and pancreatitis.

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Disclosure

The authors have no competing interest to declare.

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Keywords

Tissue mechanics; indentation; pancreatic ductal adenocarcinoma; pancreatitis; pancreatic stellate cells; cancer associated fibroblasts; collagen hydrogels

1. Introduction

Pancreatic cancer is diagnosed in about 50,000 cases annually and claims nearly 45,000 lives annually. The 5-year relative survival is 8%, and late diagnosis in more than half of the cases reduces 5-year survival to 3% [1]. Pancreatic ductal adenocarcinoma (PDAC) comprises most cases of primary pancreatic tumors, and PDAC tumors are characterized by dense regions of fibrosis surrounding clusters of cancer cells. The physical properties of these desmoplastic tumors have important, wide-ranging implications for both diagnosing and treating PDAC. The fibrosis and stiffening associated with PDAC tumors may be detectable through minimally-invasive methods like elastography and leveraged for diagnosis [2,3]. However, stiffening of the pancreas is also expected to accompany the chronic inflammation of pancreatitis, as in other inflammatory gastrointestinal diseases [4,5], which may confound diagnosis. Direct mechanical characterization of normal, PDAC, and pancreatitis tissue could inform development of non-invasive diagnosis.

Mechanical properties of the PDAC microenvironment would also inform scientific investigations of PDAC tumor growth and metastasis and translational development of therapies. PDAC is typically diagnosed in advanced stages once tumors have already developed dense desmoplasia, and the fibrotic matrix and reduced vasculature impedes delivery of chemotherapeutic agents [6,7]. Though dense fibrotic stroma may act as a barrier for diffusivity of drug agents, the deletion or suppression of stromal cells in tumors has been shown to accelerate cancer progression *in vivo* [8,9] [10]. In addition, stiffness of the tumor microenvironment in other cancers has been shown to contribute to cancer cell proliferation [11] and chemoresistance [12,13]. A better understanding of tumor tissue mechanics may improve 3D *in vitro* models of tumors for mechanistic investigations and for development and screening of therapies.

To inform both clinical practice and the development of *in vitro* models, we collected surgical specimens from human patients for direct mechanical characterization using a custom indentation system. By comparing normal, PDAC, and pancreatitis samples, we show that both elastic stiffness and viscous relaxation properties are required to distinguish between PDAC and pancreatitis. To demonstrate that *in vitro* microenvironments can be tuned to have similar properties, we characterize Living Mechanical MicroEnvironments (LiMMEs) for direct comparison to patient samples.

Our custom equipment ensured identical methods and analysis could be used on both patient samples and *in vitro* hydrogels. Comparing across methods and constitutive models can result in dramatically different values to describe stiffness. Various methods of characterizing collagen hydrogels, for example, have resulted in values for mechanical properties that span many orders of magnitude (Figure 1) [14–22]. For comparison, reported shear storage moduli were converted to elastic-like moduli assuming Poisson's ratio is 0.495

[23]. While collagen concentration and stiffness generally rise together, the potential for inconsistency in soft matter characterization across *ex vivo* and *in vitro* samples is evident. The ability of our system to quantify mechanical properties for synthetic hydrogels and tissue resections alike provides us with the advantage of direct comparison, and this work calls attention to nuances of mechanical characterization in addition to its biological findings.

2. Materials and Methods

2.1 Specimen Preparation

2.1.1. Patient Samples—Resected pancreatic tissue was generously donated by the Department of Surgery after obtaining appropriate patient consent under University of Florida Institutional Review Board-approved protocols. Tissue was collected from surgeries between 10/2014 and 02/2017. Before each surgery that was expected to result in a pancreatic tumor, indentation equipment was prepared. After surgery, a portion of the resected tissue went to pathology and to satisfy other clinical requirements. If enough tissue was left, the remaining portion was prepared for indentation testing. PDAC samples were excluded from study if patients had received chemo- or radiation therapy prior to surgery. “Normal” tissue with no histologic evidence of pancreatitis or malignancy was only available accompanying other conditions such as benign cystic lesions or duodenal adenomas. Taken together, these constraints severely limited samples available for study.

Resected tissue samples were placed in DMEM/F12–10% FBS culture media on ice in the operating room and transported to laboratory for testing. The size of the sample varied with each patient, but generally volumes ranged between 125–600 mm³. If the sample had not already been sectioned with flat, parallel surfaces, the sample was sliced in a stainless-steel matrix slicer (Zivic Instruments) to obtain a flat indentation surface approximately 2 mm thick (Fig. 1A). Samples were loosely confined in a custom holder made of silicone (Fig. 1B) and submerged with room temperature culture media (Fig. 1C). Tissue was allowed to reach ambient temperature before indentation, and all indentations were carried out no more than 2 hours after resection.

2.1.2. Collagen gels—10x DMEM (Sigma) was combined in a 1:1 ratio with HEPES (Gibco) to obtain a 5x DMEM and HEPES solution. 3.7 g of sodium bicarbonate (Fisher Bioreagents) per 160 mL was added to control pH. Rat tail collagen type-I (Corning) was diluted with 0.2% acetic acid (Glacial, Fisher Scientific) in 1x Phosphate Buffer Saline (PBS) (InvitroGen, ThermoFisher) to obtain a 4 mg/mL collagen solution. 5x DMEM was warmed to 37°C and mixed with the collagen solution at 4°C in a 1:3 ratio to obtain a 3 mg/mL collagen hydrogel precursor solution. Collagen was then allowed to thermogel at 37°C for 35 minutes before adding cell culture medium.

2.1.3. Supernatant collection—PDACs cells were isolated from patient tumors via xenograft as previously described [24]. Cells were then cultured on 24 well-plates at 5×10⁴ cells and 500 µL of 1X DMEM with 10% Fetal Bovine Serum (FBS) per well (Fig. 2). Supernatant was collected after 24 hours, spun down in 4°C centrifuge to remove debris, and stored at –80°C for no more than 2 weeks before being used to treat LiMMEs.

2.1.4. Living in Vitro Mechanical Microenvironments—TAS cells were directly isolated from patient tumors as previously described [25]. TAS cells were used between passage 7 – 11 (Fig. 2C), and 2×10^3 cells/ μL in 1x DMEM with 10% FBS and 1% Pen-strep solution was mixed with collagen precursor solution in a 1:20 ratio. Solution was aliquoted into a 96-well plate (50 μL) and allowed to thermogel at 37°C for 35 minutes. The resulting 50 μL collagen hydrogels were approximately 6.6 mm diameter by 1.5 mm thick and embedded with 5×10^3 cells per gel. “Conditioned medium” was 40% collected supernatant and 60% DMEM.

To investigate the autocrine vs paracrine signaling for fibroblast remodeling function, additional TAS-only gels were fabricated and maintained for either 24, 72, or 168 hours with PDAC supernatant. Control media was used after the designated period of transient exposure, and mechanical properties were quantified at the end of seven days in culture.

2.2 Indentation Apparatus and Force Relaxation Tests

A custom cantilever-based indenter was used to record force-displacement values during testing (Figs. 1C, 2B) [26]. A piezoelectric stage (P-628.1CD, Physik Instrumente) controls the base of a soft titanium cantilever with a 1- or 4 mm-diameter rigid tip while a custom program in LabVIEW (National Instruments) reads cantilever tip deflection with a capacitive sensor (C8S-3.2-2.0 and compact driver CD1-CD6, Lion Precision). Tip deflection is converted into force using cantilever stiffness (Fig. 3). Cantilever stiffness was calibrated to be 79.8 N/m by measuring deflection due to small weights of known mass.

Submerged patient tissue samples (1 – 3-mm thick) were indented to 10% of sample thickness (100 – 300 μm) at an indentation rate of 10 $\mu\text{m/s}$, after which the probe was kept in place for 90 – 210 seconds to allow for the tissue to achieve > 90% relaxation. Sample thickness was measured using the indenter to determine appropriate indentation depth. Samples were indented at 6 – 13 distinct locations along the length of the tissue, depending on the size of the sample and availability of flat regions.

All LiMMEs were indented to a depth of 140 μm (~10% thickness) at an indentation rate of 10 $\mu\text{m/s}$, and a time of 15 seconds was used for relaxation so that > 90% apparent relaxation was achieved. Each of three replicates was indented in three locations, yielding a total of 9 indentation results for each condition and time point. Force-displacement data from the tests (Fig. 3) were then fitted to the SLS model as described in Section 3.

2.3 Visualization of Cell Viability and Cytoskeleton

To image cell viability, 100 μL of 0.05% Calcein AM in sterile PBS (Life Technologies LIVE/DEAD® Cell Viability Assay) were added to wells and kept for 30 minutes before imaging on a Nikon Eclipse Ti-E with Andor Zyla camera.

LiMMEs were fixed using 120 μL of 4% Paraformaldehyde (PFA) in PBS for at least 2 hours to allow diffusion. Phalloidin-FITC (P5282 Sigma) – 0.1 mg/mL in methanol – was diluted 1:150 in PBS and wells were treated with 100 μL for 30 minutes, each. A 1:1000 DAPI (D1306 ThermoFisher Scientific, 1 mg/mL in diH_2O) to PBS ratio was used to treat LiMMEs for 10 minutes to label nuclei. LiMMEs were rinsed 3 times with PBS every 30

minutes for 3 hours, and Phalloidin/DAPI images were obtained using a Nikon Eclipse Ti-E microscope with a C2 confocal scanning system.

3. Theory and Calculations

3.1 Hertz Contact Model

The custom indentation system described above utilizes a spherical tip to indent the surface of a tissue at a given rate. The Hertz contact model was originally derived to calculate the elastic modulus of a flat, elastic half-space upon indentation with rigid spherical tip, so this model is typically used in such a configuration. Soft matter such as these pancreatic tissue samples violates most assumptions of the original Hertz model, though. Namely, biological materials are not purely elastic, are not homogeneous, are not frictionless and are adhesive. However, for very shallow indentations on submerged tissue, the Hertz equation (Equation 1) still fits our data well (Fig. 4A) and could be rearranged to yield a useful time-dependent transient modulus comparable to a viscoelastic “stress relaxation modulus” [27,28] (Equation 2):

$$F = \frac{4E\sqrt{R}}{3(1-\nu^2)}\delta^{3/2} \quad \text{Equation 1}$$

$$E_{transient}(t) = \frac{3 \cdot F(t) \cdot (1-\nu^2)}{4 \cdot \sqrt{R} \cdot \delta(t)^{\frac{3}{2}}} \quad \text{Equation 2}$$

F = force as calculated by tip displacement multiplied by calibrated stiffness; E = Effective elastic modulus; R = radius of spherical indenter tip (0.5 and 2 mm in these studies); δ = indentation depth calculated as stage movement minus deflection of tip; and ν = Poisson's Ratio.

Assumptions for Poisson's Ratio of gastrointestinal tissues range from 0.4 to 0.5 [29–32], and we used $\nu = 0.45$. Propagation of uncertainty calculations suggest $0.4 < \nu < 0.5$ would affect calculations $< 5\%$. Small sample thicknesses can further affect the application of the Hertz model, potentially decreasing modulus calculations up to 20% (e.g corrected modulus = $0.805 \cdot E$ using finite-thickness-sample correction for non-bonded samples [33] on worst case scenario of all tissues collected where $R = 500 \mu\text{m}$, $\delta = 100 \mu\text{m}$, and $h = 1 \text{ mm}$). Only normal samples (non-cancerous and non-inflamed) were sporadically too small to be sectioned into consistent 2 mm slices, and therefore thinner. A decrease in elasticity values from the already softest category would only benefit the distinction of the groups and overall statistical significance. The finite-thickness-sample adjustment is not tractable in combination with viscoelastic models, though.

Equation 1 has been used for a variety of other soft matter indentation studies [27,34], though many more advanced contact models also have been developed [35–39], including the also commonly used Derjaguin-Muller-Toporov and Johnson-Kendall-Roberts models.

Limitations of the Hertz model for nanoscale [34] and mesoscale [40] indentation have been previously discussed elsewhere. For simplicity, the original model proves useful to derive metrics of tissue properties for direct comparison; however, we recommend more nuanced contact models if detailed mechanical properties are desired for computational applications.

3.2 Determination of Steady-State Modulus and Characteristic Relaxation Time Parameters

Recognizing time-dependent properties of pancreatic tissue may affect translation of tissue mechanics to both basic [41] and clinical [42–44] research, we selected a simple viscoelastic model to describe the observed changes in transient modulus. In traditional stress relaxation experiments, stress relaxation modulus E_r is calculated at each time point for measured engineering stress, $\sigma(t)$, and constant imposed strain, ϵ_0 : $E_r(t) = \sigma(t)/\epsilon_0$. Similarly, we estimated the time-dependent transient $E(t)$ during our load relaxation phase using Equation 3. We then fit the relaxation portion of the total elastic modulus curve, $E(t)$, to the following exponential function of the Standard Linear Solid (SLS) model:

$$E_{transient}(t) = E_{SS} + E_K e^{-t/\tau} \quad \text{Equation 3}$$

where $E_{transient}$ is the effective, time-dependent modulus (Eqn. 1); E_{SS} is the steady-state modulus (SSM) after relaxation (Fig. 5B), also called the “aggregate modulus” [45] and “indentation modulus” [46,47] in other models; E_K is the strain-rate dependent modulus represented by the spring in the Kelvin element of the SLS model; and τ is the characteristic time decay constant defined as $\tau = \eta/E_K$, where η is the viscosity. Although non-elastic coefficients such as viscosity, porosity, and permeability may vary from model to model, we found that SSM is consistent when fitting the data to other models, so we proceed with the SLS as the simplest model that still captures viscoelastic phenomena while providing a consistent metric, the SSM.

Data were fit to the model by using MATLAB’s curve fitting tool and the nonlinear least squares method and custom equation option. Goodness of fit function was used to evaluate fits, and values were discarded if normalized mean square error was <0.9 .

4. Results

4.1 Steady-State Modulus Is Higher in Diseased Tissue than Normal

In our study, both pancreatitis (2.15 ± 0.41 kPa) and tumors (5.46 ± 3.18 kPa) have higher significantly SSM than normal tissue (1.06 ± 0.25 kPa) ($p = 0.003$ and $p = 0.002$, respectively; Fig. 6A). These pancreas elasticity trends for diseased tissue are similar to breast cancer versus normal tissue [48]. However, to use mechanical properties as a diagnostic, one must be able to distinguish between two non-overlapping states. Since SSM values overlap between inflammation cases ($1.52 - 2.80$ kPa) and PDAC tumors ($1.89 - 13.7$ kPa), elastic properties alone are likely insufficient to distinguish stiff pancreatitis from soft tumors.

4.2 Viscous Properties Follow Different Trend than Steady-State Elastic Properties

While pancreatitis and tumors both have higher SSM than normal tissue, pancreatitis samples have significantly lower time-dependent properties than both normal and cancer tissue. Both the viscosity, η , and time decay constant, τ , follow a trend for pancreatitis values to decrease and cancer values to increase (Fig. 6B–C). For viscosity, pancreatitis (63.2 ± 26.7 kPa·s) is significantly lower than both normal tissue (252 ± 134 kPa·s) and tumors (349 ± 222 kPa·s). Similarly, characteristic decay times for pancreatitis (27.6 ± 14.0 s) are significantly lower than both normal tissue (92.7 ± 46.4 s) and tumors (66.1 ± 20.8 s). For reference, 3τ is the approximate time required for the sample to relax 95%.

4.3 Paracrine Factors Induce *In Vitro* Scaffolds to Yield Tumor-Like Properties

Collagen gels without embedded cells yielded SSMs ~ 800 Pa, which was near the range of normal tissue while still allowing nutrient exchange and immunofluorescent labeling. After TAS were allowed to remodel collagen for 7 days, control LiMMEs reached SSM values (1.45 ± 0.12 kPa) within the range of normal pancreas (0.8 – 1.5 kPa). LiMMEs maintained with 60% supernatant collected from PDACs achieved higher SSM values (3.38 ± 0.146 kPa), well inside the tumor stiffness range (1.9 – 14 kPa) (Fig. 7A). Characteristic decay times of remodeled gels (representative of gel viscosity) also increase upon treatment with PDAC conditioned media (Fig. 7B). Characteristic times of PDAC and normal tissue are similar, and conditioned media induces TAS to remodel LiMMEs to characteristic times in a similar range. However, initial characteristic relaxation time of these collagen hydrogels appears too low for direct comparison of control LiMMEs ($\tau \sim 4$ s) to resected tissues (~ 10 s $< \tau < 160$ s).

4.4 Stromal Function Can Be Altered upon Transient Exposure to Paracrine Factors

To determine if shorter exposures to PDAC paracrine factors could alter fibroblast function, TAS LiMMEs were maintained with PDAC supernatant for only the first 24 or 72 hours after fabrication, followed by control media, and compared to LiMMEs maintained in conditioned media for the entire 7 days. At the end of 7 days in culture, LiMMEs treated with conditioned media (CM) during the first 24 hours are not similar to LiMMEs treated with CM for 7 days ($p = 0.0004$ for conservative Wilcoxon multiple comparisons across treatment conditions). LiMMEs treated with CM for 72 hours approach values of those treated with CM for the entire seven days ($p = 0.0521$), suggesting that stromal function is altered after only 3 days of CM exposure (Figure 8).

4.5 Cytoskeletal Fiber Assembly Increased in TAS in Response to PDAC Signals

immunofluorescence labeling of cells in LiMMEs after 7 days in culture suggests increased proliferation and spread area of TAS cells when maintained with PDAC cell supernatant (Figure 9A–B). F-Actin staining reveals increased intensity in TAS cells that have been treated with conditioned medium (Figure 9C–F), resembling activation processes seen in fibroblasts [49], cardiomyocytes [50], and valvular interstitial cells [51] caused by increased substrate stiffness.

5. Discussion

We have directly characterized human pancreatic tissue in a manner that can also be applied to engineered scaffolds for translational applications. Our characterization method has enabled us to confirm qualitative and quantitative findings that pancreatic tumors are stiffer than normal pancreas [3,52,53], but importantly, our system enables direct comparison to *in vitro* constructs that previous methods did not. Our values for elastic modulus of patient tissue were similar to direct mechanical characterization by mesoscale indentation (normal = 0.787 – 1.452 kPa and tumor = 1.885 – 13.70 kPa), and relative values for elastography showed similar trends. Shear wave elastography yielded stiffer values for tumors than normal tissue, though an order of magnitude different [52]. Endoscopic ultrasound elastography closely parallels our direct characterization, too, with pancreatitis being 100–120% stiffer than normal tissue and tumors being 200–500% stiffer [54].

Though the steady-state moduli of normal, pancreatitis, and tumor tissue populations are significantly different ($p = 0.003$), the stiffness ranges overlap for the chronic pancreatitis and PDAC groups. This is unsurprising, since inflammation accompanying many different pathologies still induces stromal activation, reduced hydraulic permeability, and fibrosis that correlate with higher tissue stiffness [55]. Since both pancreatitis and PDAC show stiffening, characterization of elastic properties alone may not be adequate for direct *ex vivo* nor *in vivo* elastography diagnostics. Focusing only on viscous properties like characteristic times will present an equivalent challenge, considering time-dependent properties from normal and cancerous tissues overlap dramatically. However, taking the two metrics into consideration simultaneously should allow a more accurate distinction between pancreatitis and pancreatic cancer: tumors present high values for both stiffness and viscosity while inflamed regions in pancreatitis are stiffer but less viscous even than normal tissue.

These data have important implications for clinical and translational research. Elastography algorithms that incorporate time-dependent, relaxation parameters have been under development since the mid 2000's and showing promising results [56–62]. Our direct characterization data suggest that detection algorithms that incorporate both elastic and viscous parameters will be able to distinguish these three categories much more robustly.

Our work also has important implications for basic research. At the bench, excitement has grown over the last decade around the ability of substrate mechanics to control cancer cell proliferation [11,63,64], migration [64–67], chemoresistance [63,68], and metastasis-like processes [69–76]. However, stiffness is not static *in vivo*, and signaling between stromal and cancer cells has been hypothesized to affect tumor mechanics. By designing a characterization system compatible with both excised samples and engineered constructs, we have demonstrated that both stromal cells and cancer-cell mediated factors are necessary for *in vitro* constructs (living *in vitro* mechanical microenvironments, LiMMEs) to reach tumor-like mechanical properties.

Though cell-embedded 3D constructs like our LiMMEs can undergo significant contraction that results in smaller condensed structures, changes in geometry do not explain changes in steady-state modulus. Other groups have used this cell compaction as its own metric for

remodeling [77,78] [79], but we used cell numbers low enough to avoid macroscopic compaction ($<10^5$ cells/ml compared to 10^6 cells/ml in some early studies [80,81]). While boundary conditions (gels attached versus unattached) matter greatly in compaction and questions of residual stress in the collagen matrix [81,82], they affect indentation characterization less. The Hertz model assumes a semi-infinite half-space and we advocate small indentations ($<10\%$ thickness), so the bottom boundary is not a critical factor. Compaction could present as reduction in thickness if gel is sticking or intentionally bound to well walls, though. Thickness reduction could cause inconsistencies in the calculated properties if indentation depth is not adjusted accordingly or highly stressed regions near well walls are indented. Since one of the conditions for our characterization method to be comparable and repeatable is for the indentation depth to be near 10% of the thickness of the sample, we measured the thickness of the samples using a calibrated z-stage stage on our microscope. Although a slight meniscus formed near the wall of the well, the thickness at the flat center of the gel used for indentations appeared unchanged at ~ 1.35 mm. Consequently, gel stiffening is not an apparent consequence of compaction.

The innate response of TAS cells to secreted factors from PDAC cells remains a focus of investigation in our group. We have recently demonstrated an intense TAS inflammatory response to PDAC exposure that is dependent on pattern-recognition receptor signaling, analogous to immune cell sensing of pathogens [83]. Here, we demonstrate that this inflammatory response is accompanied by TAS-induced mechanical remodeling that mimics conditions observed *in vivo*. The clinical implications of tumor-stromal interactions have long been known, which has prompted the development of therapies targeting TAS cells. These therapies have yielded highly variable results, though, perplexing both the clinical and scientific communities [84,85]. Further characterization and experimental replication of TAS-PDAC cell crosstalk and subsequent microenvironmental remodeling is therefore essential. These efforts will be invaluable to both understand the evolution of PDAC and discover effective therapeutic targets.

Our future work will explore the mechanisms of LiMME stiffening and changes in viscosity, which are likely to include increased cell contractility, matrix protein deposition, and matrix cross-linking. While cell proliferation after 7 days could be contributing to increased LiMME stiffness, we characterized LiMMEs with different cell numbers ~ 60 minutes after gel formation, before cells had the opportunity to spread or deposit matrix, and saw no difference in LiMME mechanical properties (Supplementary Figure A). Data from other groups suggest cancer cells are softer than the matrix itself at 400 – 900 Pa [86], confirming cell number alone is not likely to dominate stiffening. Production of hyaluronic acid (HA) is likely to affect both cell function and rate of relaxation in tissue mimics {REF}, so this matricellular protein is of particular interest for future investigations since the rate of relaxation can affect cell spreading [87] and differentiation [41]. Additional work is needed to determine which mechanisms other than proliferation predominate, though, and if any can be targeted therapeutically.

6. Conclusion

Diseased tissue is usually stiffer than its healthy counterpart, but many biophysiological processes contribute to stiffness which makes mechanics-based diagnostics challenging. Specifically, chronic pancreatitis and pancreatic cancer both present stiffer regions that can be identified through even simple palpation, but they cannot be accurately distinguished based on elasticity alone. Our SSM and viscosity measurements, through mesoscale indentation of tissue resections, suggest that consideration of both elastic and viscous properties may be more effective at distinguishing between these specific pancreatic diseases. Advances in mechanical characterization through image processing, called elastography, may be able to capitalize on this distinction between pathologies by leveraging models and algorithms that incorporate time-dependent properties such as porosity or viscosity in addition to elastic properties.

The viscoelastic properties of tissue may also affect cancer research *in vitro*. Two-dimensional culture of cells on plastic has many uses, but in many cases, it cannot recapitulate the events that occur in soft, 3D matrices that mimic *in-vivo* conditions. Our viscoelastic 3D *in-vitro* system shows the capacity to sustain pancreatic stellate cells while allowing them to respond to paracrine cues from cancer cell-conditioned medium and to modify their microenvironment.

Our characterization technique that is compatible with excised tissue and living mechanical microenvironments further demonstrates a feasible model to study cancer-stroma crosstalk in a controllable 3D system. Future investigations should identify specific mechanisms of stiffening, such as secretion of new matrix proteins and matrix crosslinking, and the effect of stiffening on cancer cell proliferation and chemoresistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge support from NCI/NIDDK U01DK108320 Consortium for the study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer (CPDPC). A.R. acknowledges support from the Robert C. Pittman Fellowship from the Institute for Cell & Tissue Science and Engineering from the Herbert Wertheim College of Engineering at the University of Florida.

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Statement of Significance

Understanding tumor-stroma crosstalk in Pancreatic Ductal Adenocarcinoma (PDAC) is challenged by a lack of stroma-mimicking model systems. To design appropriate models, pancreatic tissue must be characterized with a method capable of evaluating *in vitro* models as well.

Our indentation-based characterization tool quantified the distinct viscoelastic signatures of inflamed resections from pancreatitis, tumors from PDAC, and otherwise normal tissue to inform development of mechanically appropriate engineered tissues and scaffolds. We also made progress toward a 3D *in vitro* system that recapitulates mechanical properties of tumors. Our *in vitro* model of stromal cells in collagen and complementary characterization system can be used to investigate mechanisms of cancer-stroma crosstalk in PDAC and to propose and test innovative therapies.

Figure 1.

Reported moduli for collagen gels range three orders of magnitude for small concentration variations. Different papers reporting modulus to denote elastic component of mechanical properties use various names, e.g. Young's Modulus, Elastic Modulus, Apparent Modulus, and Storage Modulus, and cover wide-ranging values for similar concentrations and formulations. Precise test conditions are described in respective reports [14–22], but notably, effective modulus is different at low (0.1 mm/min, 16A) and high (1 mm/min, 16B) displacement rates [16]. For comparison, reported shear storage moduli were converted to elastic-like moduli assuming Poisson's ratio is 0.495 [23].

Figure 2. Samples of resected pancreatic tissue were indented using custom indentation device. Large samples as in the example tissue shown were sliced to obtain a flat top surface (A) and placed into a custom silicone well (B). Well was flooded with room temperature media to fully submerge sample during indentation (C).

Figure 3.

(A) Xenograft PDAC tumors are grown in mice to expand and obtain pancreatic cancer (PDAC) cells, while tumor-associated stromal (TAS) cells are directly isolated from resected tumor. Conditioned medium is collected from PDAC cells and used to maintain Living Mechanical MicroEnvironments (LiMMes), collagen hydrogels embedded with TAS. (B) LiMMes are indented by moving the piezo-electric stage (1) down. Contact between the spherical probe (2) and the sample causes the cantilever (3) to deflect, while the capacitive sensor's (4) deflection measurements are used to calculate indentation forces. (C–D) TAS and PDAC cells cultured on plastic surfaces and labeled to visualize F-Actin (green) and nuclei (blue). Scale bars = 100 μm

Figure 4.

Graph showing indentation depth (top dashed orange line) and the normal force exerted by the tissue (bottom solid blue line) for a representative tumor sample. The central relaxation portion (labeled between 30–240 s) was used to calculate steady-state modulus as described in Section 3.

Figure 5.

Hertz fit to quasi-static loading (A) yields comparable elasticity results to SSM obtained through relaxation experiments (B). However, unlike quasi-static loading, relaxation curves fitted to different constitutive models also allow quantification of time-dependent properties like viscosity.

Figure 6.

Elastic properties of diseased tissue follow different trends than viscous properties. (A) Steady-state modulus (SSM) of pancreatic ductal adenocarcinoma tumors (PDAC, “cancer”) is significantly higher than pancreatitis ($p < 0.001$) and normal ($p = 0.002$) resected tissues. (B) Viscosity, or liquid-like property, of pancreatitis tissue is significantly lower than PDAC ($p < 0.001$) and normal ($p = 0.003$) resected tissue. (C) Characteristic time of relaxation follows the same trend as viscosity. For definition of viscosity and characteristic time, see text and notes for Eqn. 3. P-values were calculated using a Wilcoxon nonparametric multiple comparisons test for each property. Box plots indicate mean, quartiles, and range. ^Outlier of 13.7 kPa not shown; †Outlier of 3400 kPa·s not shown

Figure 7.

LiMMEs reach SSM values within the ranges of normal and tumor tissue when maintained with control and conditioned medium, respectively (A). Characteristic Relaxation Times (B) show a similar increasing trend when treated with cancer cell supernatant, while controls remain mostly unchanged. ^Outlier of 13.7 kPa not shown; †Outlier of 157 s not shown.

Figure 8.

SSM values after 7 days of culture for TAS LiMMES. LiMMES were treated with PDAC cell supernatant (conditioned media, CM) for 24, 72, or 168 hours after fabrication as indicated and maintained in control media until characterization on day 7 (indicated by open circles). Untreated control LiMMES on Day 7 are repeated here from Figure 7 for reference (open boxes), as are LiMMES treated with CM for 72 hours and characterized at 72 hours (grey boxes). Transient CM exposure for 72 hours seemed to induce lasting stromal transformation that accelerated remodeling for the entire time period.

Figure 9. Visualization of tumor-associated stroma (TAS) cells in Living Mechanical MicroEnvironments (LiMMEs) on Day 7. (A, B) Calcein AM immunofluorescent plasma label suggests an increase in TAS proliferation and spreading in treated LiMMEs. (C–F) Labeled actin and nuclei of TAS in LiMMEs after 7 days in culture. Confocal images (C–D) reinforce 3D nature of gels, with nuclei appearing out-of-plane, and fluorescence images (E–F) illustrate actin fibers spanning various focal depths.