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Separation of Solid Stress From Interstitial Fluid Pressure in Pancreas Cancer Correlates With Collagen Area Fraction

Elevated total tissue pressure (TTP) in pancreatic adenocarcinoma is often associated with stress applied by cellular proliferation and hydrated hyaluronic acid osmotic swelling; however, the causal roles of collagen in total tissue pressure have yet to be clearly measured. This study illustrates one direct correlation between total tissue pressure and increased deposition of collagen within the tissue matrix. This observation comes from a new modification to a conventional piezoelectric pressure catheter, used to independently separate and quantify total tissue pressure, solid stress (SS), and interstitial fluid pressure (IFP) within the same tumor location, thereby clarifying the relationship between these parameters. Additionally, total tissue pressure shows a direct correlation with verteporfin uptake, demonstrating the impediment of systemically delivered molecules with increased tissue hypertension. [DOI: 10.1115/1.4036392]

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death, marked by a 6 month median survival and a 5 year survival rate of approximately 7% [1]. Primary symptoms of the disease, such as abdominal pain and jaundice, are caused by mass effects rather than disruption of the exocrine and endocrine function [2], leading to late-stage diagnosis and ~80% of patients are ineligible

for surgical resection [3]. Due to aggressive localized invasion with micrometastases and apparent resistance to chemotherapy [4], patients who undergo pancreaticoduodenectomy have a 5 year survival rate of at best 27% [1]. The poor prognosis of the disease has been associated with tumor cell resistance and multiple drug resistance; however, reduced vascular patency within the solid tumor and an enhanced desmoplastic reaction have become the primary focus as both limit the delivery of drug molecules into the tumor interstitium [5]. The causal role of stromal development and increased interstitial pressure upon drug delivery has been an issue of intense research, to better understand the limitations imposed by the biophysical microenvironment, and the origins

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Fig. 1 Proposed description of the extracellular matrix component contributions that limit drug distribution in pancreatic cancer. The extracellular matrix component contributions that limit drug distribution and instigate widespread vascular collapse is outlined in (a). Excessive extracellular component deposition coupled with cellular proliferation results in growth-induced solid stress, causing reduced vascular and lymphatic patency and elevated interstitial fluid pressure, thereby, limiting convective transport of large macromolecules into the tumor interstitium (b).

of vascular collapse in these tumors. In this study, a new approach to measure interstitial fluid pressure and solid stress was demonstrated with a modified pressure transducer. These data grant insight into the mechanisms of collagen interaction with pressure and vascular patency within pancreatic tumors.

A proposed description for the biophysical features that limit drug distribution in pancreatic cancer is outlined in Fig. 1(a). Desmoplasia is a hallmark of pancreatic cancer, which occurs as pancreatic stellate cells that are activated from quiescent to a myofibroblast-like phenotype and secrete excess extracellular matrix (ECM) components [6], such as glycosaminoglycans (i.e., hyaluronic acid) and collagen [5]. This dense ECM provides a facilitatory tumor microenvironment for cancer cell migration, tumor growth, and resistance to apoptosis [7], contributing to interstitial hypertension and growth-induced solid stress [8]. Stylianopoulos et al. [8] defined solid stress (SS) as stress applied to structural elements of the tumor caused by the migration and proliferation of cancer cells, composed largely of collagen and hyaluronic acid. These SS elements store and transmit stress directly by compressing cancer cells and stromal cells and indirectly by compressing blood and lymphatic vessels [9], as illustrated in Fig. 1(b). Provenzano et al. [5] has shown that up to 75% of vessels within autochthonous pancreatic ductal adenocarcinoma (PDA) in mice were collapsed, illustrating the inherent barrier to systemically delivered therapies. Chauhan et al. [10] has shown that both hyaluronic acid and collagen contribute to reduced vascular patency in tumors with enhanced desmoplasia; however, collagen played the dominant role in vessel collapse.

Collapsed and nonfunctional lymphatics resulting from elevated interstitial hypertension prevent fluid and plasma macromolecules from draining out of the tumor, thereby resulting in free fluid accumulation in the tumor interstitium and elevated interstitial fluid pressure (IFP) [11]. As IFP increases to the level of microvascular pressure (MVP), convective transport becomes negligible, leaving diffusion as the primary means for transvascular to interstitial transport of drug molecules into pancreatic tumors.

One critical area in this field, inspiring significant debate among researchers, is defining hydrated hyaluronic acid as a component of IFP or growth-induced SS. Hyaluronic acid is a highly negatively charged glycosaminoglycan that is overexpressed in pancreatic cancer tissue; a 12-fold increase of hyaluronic acid content compared to normal pancreas was shown in a separate study [12]. Each negative charge within hyaluronic acid aggregate requires a counter-ion to achieve electro-neutrality, resulting in elevated osmotic pressure that drives fluid into and out of the aggregated glycosaminoglycan: this is referred to as Donnan equilibrium pressure [13]. Elevated osmotic pressure due to Donnan equilibrium pressure and electromechanical repulsion by the negative charges of aggregated glycosaminoglycan [14] results in swelling of the tissue matrix. The integrity of the collagen matrix

elastically resists the swelling force, producing an equal stress to the Donnan equilibrium pressure to maintain a constant tissue volume. This equilibrium stress is referred to as prestress in literature [15]. The solid collagen matrix counterbalances the large absorption pressure resulting from this osmotic swelling, thereby limiting fluid absorption and achieving tissue homeostasis. Therefore, the pressure elevation is an indirect measurement of the internal stress imposed by the collagen–proteoglycan matrix.

DelGiorno et al. [16] describes the observed elevated pressure in PDA, measured values ranging from 75 mmHg to 130 mmHg [5] using a Millar Mikro-tip piezoelectric pressure transducer probe (Millar Inc., Houston, TX), as a direct measurement of IFP. In their model, IFP is divided into a two-phase fluid model for tissue, comprised of a “mobile” fluid, representing free fluid within tissue interstitium, and an “immobile” fluid, representing fluid bound within hyaluronic acid. The two-phase fluid model, free fluid and immobile fluid, was first introduced by Brace and Guyton [14] to explain negative interstitial pressures measured using implantable hollow capsules, which solely measure IFP. Their work concluded that both osmotic and hydrostatic pressures arise from highly negative proteoglycans (primarily hyaluronic acid), and the combination of these effects results in an absorption pressure within tissue, driving fluid into the proteoglycan–collagen matrix to establish a charge balance. Brace and Guyton defines osmotic force as the transfer of fluid between the interstitium and the proteoglycan–collagen matrix (fluid component) and the hydrostatic force as mechanical recoil resulting from compressed elastic elements in the proteoglycan–collagen matrix (solid component). Since no practical measurement techniques exist to quantify osmotic force and hydrostatic force individually, Brace and Guyton grouped these terms into a single variable within a modified Starlings equation for interstitial fluid transport: $p_c - \Pi_c = p_{if} - \Pi_{if} + p_{don}$, where c stands for capillary, if stands for interstitial fluid, and don stands for Donnan force.

The modified Starlings equation only entails fluid transport mechanisms and falls short in describing the solid structural interactions which are pivotal in determining the tissue pressure measurements conducted with a Millar piezoelectric sensor used in their study [5,16,17]. The piezoelectric sensor is indiscriminative in the mechanism that applies stress on the transducer, and therefore, requires a model that describes both fluid and solid interactions within tissue. Chauhan et al. [18] instead associated the elevated pressure measured by the piezoelectric pressure transducer as a combination of both IFP and SS. In their model, collagen fibrils provide tensile strength and resist the stress imposed by migrating and proliferating tumor cells [9]. Hyaluronic acid imbibes water molecules and provides compressive resistance to cancer cell proliferation. Therefore, Chauhan et al. [18] asserted that the piezoelectric probe measures a combination of SS, initiated by cellular proliferation, and IFP within the interstitium, defined as total tissue pressure, TTP (Fig. 1(a)) and illustrated in

Fig. 1(b). A similar representation of total pressure measured as a combination of mechanical stress and fluid pressure dates back to Terzaghi [19] in 1923 in the pivotal work in 1D consolidation theory used to quantify the displacement of moving soil. In Terzaghi's representation, the mechanical stress was referred to as effective stress and fluid pressure referred to the mean fluid pore pressure.

The Millar piezoelectric pressure transducer used in this study, as well as others [5,16,17], has been referred to as a gold standard in measuring IFP in both positive and negative pressure regimes [20]. However, the inherent nature of a piezoelectric device prevents separation of the IFP from SS, whether resulting from an immobile fluid phase (expanding hyaluronic acid), free fluid accumulation, collagen, or cellular migration and proliferation. This has been illustrated in numerous studies [8,16,18,21], where the piezoelectric sensor has measured pressure considerably larger than the wick-in-needle technique [21]. Currently, quantification of IFP and SS is limited to independent measurements for each parameter. Chauhan et al. [18] suggested that the best method to measure interstitial fluid pressure only was the wick-in-needle technique [22], with which a mean IFP of 11.8 mmHg was measured in PDA [18]. To measure SS, Stylianopoulos et al. [8] developed a method of cutting excised animal tumors and estimating SS from the observed deformation of the tumor. A technique that can achieve both, measuring IFP and SS at the same location in tissue, is needed to provide insight into the relationships between IFP and SS in respect to patency of vasculature and drug uptake.

In this study, a conventional piezoelectric transducer was used to measure TTP within pancreatic tumors. TTP is the combination of interstitial stress propagating from SS from tissue components, such as collagen, hyaluronic acid, and cellular migration and proliferation, and IFP within the tissue interstitium [18], $TTP = SS + IFP$. We modified a piezoelectric transducer to directly measure IFP in the same tumor location, thereby indirectly quantifying SS. The SS component of TTP is hypothesized to reduce vascular patency and drug uptake in pancreatic tumors. Measurements of TTP were correlated to ex vivo analysis of vascularity, vascular patency, and in vivo drug delivery of verteporfin in the same tissues.

Materials and Methods

Animals and Tumor Models. The tumor line used for this study was a human pancreatic adenocarcinoma xenograft, AsPC-1. A total of 24 female athymic nude rats, between 6 and 8 weeks of age, were implanted via injection of 1×10^6 cells, in 50% Matrigel (BD Biosciences, San Jose, CA) and 50% complete media, subcutaneously into the right flank and orthotopically directly into the pancreas. Two tumor locations were incorporated into this study as the host-tumor microenvironment has previously been shown to influence tumor growth, metastatic potential, and efficacy of systemic treatments [23]. The tumors were allowed to grow for a period of 4–6 weeks, until reaching an average diameter of 10–15 mm for orthotopically grown tumors and 5–8 mm for subcutaneously grown tumor. All animal procedures were conducted under the protocol approved by the Dartmouth Institutional Animal Care and Use Committee (IACUC).

Total Interstitial Pressure Measurements. Conventional pressure measurements were performed using a Millar Mikro-tip piezoelectric pressure catheter (model SPR-671, 0.47 mm diameter) with a dynamic pressure range from -50 mmHg to 300 mmHg and a nominal sensitivity of $5 \mu V/V/mmHg$. The pressure catheter output was amplified using an AD8221 precision instrumentation amplifier (Gain = 991) and filtered using a first-order low-pass filter ($f_{cutoff} = 0.1$ Hz). Data were acquired using VERNIER LABPRO data acquisition unit (Vernier Software and Technology, Beaverton, OR) and the data were recorded using VERNIER LOGGERPRO3 software (acquisition rate = 1 Hz). Prior to

each experiment, the system was calibrated in a 60 inH₂O (≈ 112 mmHg) water column at a temperature of 37 °C.

To place the pressure catheter into tumor tissue, a 23-gauge needle was first introduced into the tissue and the Millar catheter was quickly placed into and then removed from the track created by the needle. The probe was removed and reinserted several times to avoid adherence to the surrounding tissue. The Millar sensor was approximately located at the center of each tumor. Data were collected for a minimum of 10 min for each tumor or until a constant pressure value was observed.

Interstitial Fluid Pressure Measurements. To isolate interstitial fluid pressure from total tissue pressure, the Millar pressure catheter was covered with 24-gauge (ID = 0.56 mm) polytetrafluoroethylene (PTFE) tubing (STT-24-10, Small Parts). A 30-gauge hypodermic needle was used to initially poke slits into the PTFE cover to allow transmission of interstitial fluid pressure to the fluid within the PTFE cover. The Millar catheter and PTFE cover were placed into a water bath ($T \approx 37$ °C) until water surrounded the probe within the tubing. Prior to each experiment, the system was calibrated in a 60 inH₂O (≈ 112 mmHg) water column at a temperature of 37 °C.

The method for measuring IFP and SS using the PTFE cover was validated with a solid stress phantom made from silicone rubber (Smooth-on Inc., Macungie, PA), as shown in Fig. S1(a) (SI Materials and Methods), which is available under the “[Supplemental Materials](#)” tab for this paper on the ASME Digital Collection. The Millar probe and PTFE cover measured a negligible pressure change (~ 3 mmHg) when inserted into this solid stress phantom; however, the measured pressure significantly increased (~ 120 mmHg) when the cover was removed from the Millar probe, see Fig. S1(b), which is available under the “[Supplemental Materials](#)” tab for this paper on the ASME Digital Collection. This illustrated that minimal SS was applied to the piezoelectric element, while the PTFE cover is placed over the Millar probe. Correct measurement of IFP was confirmed in a 60 in water column ($T = 37$ °C). An identical pressure was measured using the Millar pressure catheter with and without the PTFE cover, indicating minimal interference to fluid pressure transmission, see Fig. S1(d), which is available under the “[Supplemental Materials](#)” tab for this paper on the ASME Digital Collection.

Quantifying Tissue Parameters. Tumor sections were embedded in paraffin, thinly sectioned ($4 \mu m$), and stained with Masson's trichrome stain, to quantify collagen content. Sections were viewed at $10\times$ magnification and eight color images were captured for each tumor section. Images were converted from RGB to CIE-Lab color space (details in SI Materials and Methods, which is available under the “[Supplemental Materials](#)” tab for this paper on the ASME Digital Collection), a 3D color space where X-axis and Y-axis represent the complete color gamut and Z-axis represents lightness of color. A k -means clustering algorithm was implemented in MATLAB to partition all pixels represented in the image into k clusters. The clusters represent centroids that surround a mean value, where the mean values are a maximum Euclidean distance away from each other, allowing for maximum color segmentation. The result of this k -means clustering algorithm in MATLAB was used to partition the original image into three subimages, one solely representing Masson's trichrome stain. Collagen area fraction (%) was quantified by calculating the number of pixels stained with Masson Trichrome compared with the total number of pixels in each image. The mean collagen area fraction (%) for eight images is displayed.

Endothelial cell surface receptor CD31 was used as a marker for tumor vasculature structure, using immunostaining for anti-CD31 (BD Biosciences) on frozen ($4 \mu m$) sections, using appropriate tissue controls as routinely completed in our research pathology Share Service of the Norris Cotton Cancer Center, as described in Ref. [24]. Sections were viewed at $10\times$ magnification

Fig. 2 Novel technique for measuring individual pressure contributions in pancreatic cancer. A Millar Mikrotip pressure catheter (a) and a modified cover (b) were used to measure total tissue pressure (TTP) as a combination of solid stress (SS) and interstitial fluid pressure (IFP) (c) and IFP only (d). The modified attachment was designed to easily retract, thereby measurement of IFP and TTP were obtained without removing the probe from the tumor location, as illustrated in (e). In this tumor, the modified Millar pressure sensor was placed in water from time = 0 to 2 min and placed in the tumor from time = 2 to 12 min. The modified cover was removed at time = 12 min and Millar pressure measurements (no PTFE cover) continued until time = 22 min.

and eight color images were captured for each tumor section. The images were randomly selected across the tissue slice and pattern was consistent with all tumor slices. Vascular structure was quantified as a vessel area fraction (%) by calculating the number of pixels stained with anti-CD31 compared with the total number of pixels in each image. The mean vessel area fraction (%) from eight images was reported. The mean vessel area fraction (%) was compared between the orthotopically grown tumors and subcutaneously grown tumors using a Welch *t*-test. The Welch *t*-test is a *t*-test that used to analyze data with equal or unequal sample sizes and unequal variances.

Vascular patency was measured using an injected stain, DiOC₇ (ThermoFisher Scientific, Waltham, MA) administered intravenously at a concentration of 1 mg/kg, 1 min prior to sacrifice, as demonstrated in a previous study [25]. The tumor was excised and frozen. Sections were viewed at 10× magnification and eight color images were captured for each tumor section. Patent vessel area fraction (%) was quantified by calculating the number of pixels stained with DiOC₇ compared with the total number of pixels in each image. The mean patent vascular area fraction (%) from eight images was reported. The mean patent vascular area fraction (%) was compared between the orthotopically grown tumors and subcutaneously grown tumors using a Welch *t*-test.

Quantifying In Vivo Dosimetry. Verteporfin (Sigma-Aldrich, St. Louis, MO), a photosensitizer used predominately for photodynamic therapy [26], was used as a contrast agent in this study. Verteporfin was administered intravenously at a concentration of 1 mg/kg. Two minutes postinjection, optical measurements were acquired at three locations on the subcutaneous tumor and four locations on orthotopic tumors of each rat, using a custom designed fiber-optic spectroscopic device [27]. This device

sequentially measured white light reflectance (between 450 and 900 nm), and fluorescence emissions (in the range 650–800 nm) resulting from excitation with laser light at 635 nm. The sampled fluorescence was used to quantify verteporfin content within the sampled tissue volume by an analysis method that included the following steps: calibration of the raw spectra to a reference phantom (1% intralipid phantom), correction for the influence of optical properties using the white light spectroscopy measurements, and spectral fitting to separate verteporfin fluorescence emissions from other sources of fluorescence such as tissue autofluorescence and photoproducts [27]. Error analysis of spectral fitting was used to ensure robustness of optical parameters, with measurements made in either poor contact with the tissue or placed in a blood pool (defined as <20% blood volume) identified and removed from analysis. Optical measurements were collected up to 30 min postinjection.

Results

Total Tissue Pressure and Interstitial Fluid Pressure Measured in the Same Tumor Location. The Millar Mikrotip pressure catheter, Fig. 2(a), used in this study outputs a voltage corresponding to applied stress on the piezoelectric element of the transducer. In tissue, this stress propagates from SS from tissue components, such as collagen, hyaluronic acid, and cellular proliferation, and IFP within the tissue interstitium to the Miller piezoelectric element (i.e., $TTP = IFP + SS$), as illustrated in Fig. 2(c). IFP, approximately equal to microvascular pressure [11], eliminates convective molecular transport into tumors. SS has been shown to inhibit diffusive transport [28] and instigate widespread vascular collapse within tumors [29]. We hypothesized that measuring both IFP and TTP would provide essential

Fig. 3 Correlation between collagen area fraction and total tissue pressure (TTP). Millar Mikrotip (SPR-671) pressure catheter was used to measure pressure in AsPC-1 tumors and this was compared to collagen area fraction analyzed ex vivo. A strong correlation was observed between collagen area fraction and TTP using a linear regression fit ($R^2 = 0.799$; $*p < 0.001$). *P*-value was calculated under the null hypothesis no correlation between TTP and collagen. The *y*-intercept was 23 mmHg, approximately equal to microvascular pressure (MVP) found in literature [32]. Interstitial fluid pressure (IFP) will not exceed MVP indicating values of $TTP > IFP$ represent solid stress (SS). Comparison between measured values of TTP (mean = 48.8 mmHg) and IFP (mean = 12.8 mmHg) was made by a Welch *t*-test ($**p < 0.001$) for five tumors.

information on localized molecular uptake and vascular patency. Our group isolated IFP from TTP by using polytetrafluoroethylene (ID = 0.56 mm) tubing as a protective cover, surrounding the Millar pressure catheter, as shown in Fig. 2(b). The PTFE cover was designed to eliminate contact of ECM components on the piezoelectric element of the transducer and allow transmission of IFP into the fluid surrounding the probe, Fig. 2(d). The PTFE cover was 5 in in length and could be effortlessly removed from the probe without changing the position of the Millar sensors within the tumor. In this way, both IFP and TTP could be measured within the same location.

Figure 2(e) illustrates the usefulness of our method. At time = 2 min, the Millar probe, with PTFE cover, is inserted into the tumor interstitium. IFP was measured for 10 min with a mean value of 9.4 mmHg. At time = 12 min, the PTFE cover was carefully retracted from the sensor region of the Millar probe, while taking care to preserve the probe location within the tumor. From time = 12 min to 22 min, a mean TTP of 59.2 mmHg was measured. SS was approximated from the difference between TTP and IFP to be 49.8 mmHg ($TTP = IFP + SS$). Slight temporal variations (± 10 mmHg) were observed in our measurements of TTP for the time scale of 10 min. Such variations can be explained by disturbance of the tissue microenvironment due to the introduction of our piezoelectric sensor and relationship of the viscoelastic tissue elements, as well as temporal variations in blood flow and oxygenation, which has been measured in the range of 10 mmHg by Dewhirst et al. [30] in R3230AC mammary carcinoma.

Elevated Collagen Area Fraction Increases Compressive Stress Within Pancreatic Tumor Interstitium, Thereby, Increasing Total Tissue Pressure. Increased deposition of collagen within ECM is a byproduct of an enhanced desmoplastic reaction within pancreatic tumors. Collagen is a structural component of the ECM that possesses both viscous and elastic properties, exhibiting deformation that depends on the rate at which it is strained [31]. The larger quantity of collagen would provide a stronger barrier to cellular proliferation and expanding hyaluronic acid, thereby increasing SS within the tumor interstitium. Our group hypothesized that total collagen content within the tumor would directly correlate with TTP measured with the Millar

pressure catheter. This hypothesis has been suggested in related manuscripts [8,10,16]; however, not empirically proven until now.

The results of this study illustrated a strong correlation between TTP and collagen area fraction (%), Fig. 3, in 24 AsPC-1 xenograft tumors. Mean values for each tumor were fit using linear regression with $R^2 = 0.799$ ($p < 0.001$). These findings coincide with the work of Chauhan et al. [10], which showed degradation of collagen area fraction using an angiotensin inhibitor, losartan, resulted in a reduction of SS. The mean value of TTP presented within this study is 48.8 mmHg, coinciding with findings of other pancreatic xenograft tumors studied with the Millar piezoelectric pressure catheter [21]. The *y*-intercept (23 mmHg) approximately equaled values of microvascular pressure (MVP) found in literature [32]. This would suggest that IFP and MVP are approximately equal and IFP is unaffected by collagen area fraction. Stylianopoulos et al. [8] showed that IFP was uncorrelated with growth-induced stress in another study.

Localized Total Tissue Pressure Measurements Showed Minimal Affect on Total Vascular Density, Vascular Patency, and Verteporfin Uptake in Pancreatic Tumors. Our group hypothesized that SS, measured as a function of TTP, would correlate with vascular structure, vascular patency, and vascular perfusion within the tumor interstitium. Our results showed that quantification of tumor structure, by the anti-CD31 immunostain (Fig. 4(a)) in the central region of the tumor (excluding tumor periphery), showed no apparent correlation with increasing values of TTP. However, orthotopically grown tumors appeared statistically more vascularized than the subcutaneous counterpart, $p = 0.05$ (Fig. 4(b)). The AsPC-1 human pancreatic adenocarcinoma xenograft is known to have limited expression of the cyclooxygenase (COX-2) protein [33], a protein that promotes angiogenesis; however, this result indicates that the host-tumor interaction has a strong influence on the vascular structure within a tumor [23].

Quantification of tumor vascular patency, by the DiOC₇ stain (Fig. 4(c)) in the central region of the tumor (excluding tumor periphery), also did not change with increasing values of TTP. There was no apparent correlation between orthotopically and subcutaneously grown tumors (Fig. 4(d)). Mean values of DiOC₇ are approximately equal and/or slightly larger than anti-CD31. The quantification of both DiOC₇ and anti-CD31 were area fractions of positive stained pixels compared with total pixels. The larger values of DiOC₇ simply indicate possible diffusion of DiOC₇ out of tumor vasculature during 1 min of systemic circulation, thereby resulting in a larger area fraction. By normalizing the median values of DiOC₇ by the median value of anti-CD31 stained area, subcutaneous grown tumors were 10% less perfused than orthotopic grown tumors.

The mean values of verteporfin uptake using a fiber probe-based fluorescence quantification technique [27] (Fig. 4(e)) were acquired for subcutaneous and orthotopic locations and compared with measurements of TTP for each tumor. The results showed a statistically significant decrease in verteporfin uptake in orthotopic tumors ($R^2 = 0.46$; $p < 0.04$) and a no correlation with the subcutaneous tumors ($R^2 = 0.43$; $p > 0.1$). Verteporfin uptake was also elevated in orthotopic tumors compared with subcutaneous tumors (Fig. 4(f), $p < 0.01$), indicating an elevated global vascularization within orthotopic tumors.

Discussion

This study introduced a new modification to a conventional piezoelectric pressure catheter, used to independently separate and quantify TTP, SS, and IFP within the same tumor location, thereby clarifying the relationship between these parameters. Del-Giorno et al. [16] indirectly demonstrated the significance of our PTFE cover for the Millar pressure sensor by using two pressure measurement techniques and an extracellular matrix (ECM)

Fig. 4 Quantitative investigation on drug uptake in pancreatic tumors. Vascular structure and vascular patency were analyzed ex vivo with anti-CD31 immunostain (a) and DiOC₇ stain (c), respectively. A statistically significant relationship existed between the orthotopic tumor location (12 tumors included) and subcutaneous tumor location (eight tumors included) for the anti-CD31 (b) stain ($p < 0.05$) but no apparent correlation with the DiOC₇ stain (d); comparison was made by a Welch t -test. A statistically significant correlation between orthotopic location of verteporfin uptake and TTP (e) was determined using linear regression fit ($R^2 = 0.46$, $p < 0.04$). P -value was computed under the null hypothesis of no correlation between fluorescence signal and TTP. A statistically significant relationship existed between the orthotopic tumor location (f) for verteporfin uptake compared with the subcutaneous tumor location was determined by a Welch t -test ($p < 0.01$). The p -values were computed against a null-hypothesis that no correlation exists. A zero-slope line indicated no correlation, as illustrated with the subcutaneous tumor location.

degrading enzyme. In this study, pressure measurements were taken in AsPC-1 xenograft tumors using a Millar pressure sensor (measures TTP) and wick-in-needle technique [22] (measures IFP) with values of 42 mmHg and 22 mmHg, respectively. Upon systemically administering PEGPH20, an enzyme that degrades interstitial hyaluronic acid, pressure measurements using the Millar pressure catheter, and wick-in-needle decreased, converging at a pressure of IFP $\approx$ 11 mmHg within 120 min. This indicated that degradation of interstitial hyaluronic acid reduced stress on collagen fibrils within the ECM, thereby lowering the SS contribution and allowing TTP to approach IFP. The use of a PTFE cover over the unprotected Millar pressure catheter achieves quantification of IFP, TTP, and SS, in a single location (Fig. 2(e)), without implementing the complicated procedure used by DelGiorno et al. [16].

In attempt to isolate IFP from the Millar pressure catheter measurement, DelGiorno et al. [16] developed a technique referred to as piezoelectric catheter-in-needle (PC-N). The Millar pressure catheter was placed within a needle with a side-slit, similar to the wick-in-needle (WN) technique [22], and pressure measurements were compared between the piezoelectric catheter (PC) and PC-N. In this study, the PC and PC-N measured intratumoral pressure to be 32 mmHg and 31 mmHg, respectively, while the wick-in-needle measured pressure to be 25 mmHg. No difference in pressure was measured between PC and PC-N, and the pressure values were consistently larger than the WN technique, indicating that the PC-N fails to isolate external stress, i.e., solid stress, from the measurement of the probe. The modified piezoelectric catheter presented in this study illustrates significant differences in pressure with and without the PTFE attachment, Fig. 3, and the values of IFP coincide with those measured using the wick-in-needle technique for other pancreatic xenografts [21]. Additionally, the modified piezoelectric technique was designed as a removable attachment, achieving measurements of both IFP and TTP within the same tumor location and minimizing disturbance to the tumor microenvironment, as only a single injection site is required to measure both variables.

The results presented in Fig. 3 illustrated a linear correlation of collagen area fraction with TTP, which represents the equilibrium

between the force applied to the collagen matrix and the resulting mechanical deformation. The resistance of the collagen-matrix to mechanical deformation can be measured through an elastic modulus, which represents the relative mechanical strength of the collagen-matrix and the ability of the collagen matrix to impede osmotic swelling and cellular proliferation within the tumor interstitium. The mechanical properties of collagen and other ECM components are critical in tissue engineering for ECM scaffold development and have been thoroughly investigated. Roeder et al. [34] investigated mechanical properties of type I collagen solutions with increasing collagen concentrations (0.3–3 mg/mL). The results illustrated a linear increase in elastic modulus for solutions with increasing collagen concentrations. Provenzano et al. [35] investigated collagen gels imbedded with mammary epithelial cells. Provenzano combined the results from Roeder [34] with their own and showed a cubic relationship between elastic modulus and collagen concentrations ($R^2 = 0.994$). The results presented in Fig. 3 were fit using a third-order polynomial and a negligible improvement in goodness of fit was observed ($R^2 = 0.8$). The heterogeneity of collagen within pancreatic tumors yielded a global (entire tumor) correlation between collagen and localized measurement of TTP; however, a more localized quantification of collagen would be required to pick out higher-order trends.

Figure 3 suggests that degradation of collagen within the ECM of pancreatic tumors would reduce SS and lower TTP. Gade et al. [36] illustrated this by measuring TTP using a Millar Mikrotip pressure catheter in HT29 human colon adenocarcinoma xenografts implanted subcutaneously in male nude mice. Upon systemically administering 100 μ L collagenase, the mean TTP reduced to 64% of the control value, 120 min postinjection. Collagenase has also shown a dose-dependent reduction in IFP and MVP in OHS human osteosarcoma xenografts grown subcutaneously in female athymic BALB/nu/nu mice [37]. A reduction of 45% and 26% of IFP was measured using the wick-in-needle technique upon systemically administering 0.1% and 0.01% collagenase, respectively. Administration of 0.1% collagenase reduced MVP by a maximum of 60% 40 min postinjection, recovering to

control 100 min postinjection, and a two-fold increase in murine monoclonal antibody (IgG 2b) was measured in the tumor. The brief reduction in MVP was explained as an increased vascular area, up to a three-fold increase was shown in a separate study [38], which increased perfusion and reduced the drug delivery barrier characteristic to tumors.

Inhibition of hedgehog (Hh) signaling pathways has been shown to reduce stromal load by decreasing collagen I production in pancreatic tumors [39]. Introduction of IPI-926 hedgehog inhibitor showed reduction in stromal development, a statistically significant increase in vascularization and 60% increase in gemcitabine uptake in pancreatic tumors. Stylianopoulos et al. [8] also showed that application of saridegib IPI-926 resulted in a reduction of growth-induced solid stress, indicating reduction of collagen results in reduction in tissue hypertension. These results illustrate the usefulness of quantifying stromal content and collagen area fraction as the dense ECM of pancreatic tumors provide an impending barrier of resistance in pancreatic tumors and collagen area fraction could be used to predict therapeutic benefits of systemically delivered therapies.

The viscoelastic behavior of collagen transmits pressure in respect to resisting mechanical deformation; therefore relieving the force applied to the collagen matrix conceivably could reduce TTP without degrading the collagen content within the tumor. Provenzano et al. [5] demonstrated this by administering PEGPH20 to autochthonous pancreatic tumors and measuring greater than a four-fold reduction in TTP using a Millar pressure catheter and a ~71% increase in discernable vessel diameter. Degradation of interstitial hyaluronic acid mobilized bound water molecules and reduces solid stress applied to the surrounding collagen matrix, thereby decreasing TTP and increasing vascular perfusion. Tufto et al. [38] showed similar results in increased vascular area upon administering hyaluronidase and suggest that application of both hyaluronidase and collagenase could drastically improve systemically delivered therapies in highly fibrotic tumors.

The results in Fig. 4(d) show no apparent difference between TTP and vascular patency, contrary to related studies [5,36–38]. In these related studies, increased vascular perfusion values were measured as averaged values for tumors pretreatment and post-treatment after applying ECM degrading enzymes and were not analyzed on a tumor-to-tumor basis as in Fig. 4(d). Measured values of TTP were less than microvascular pressure; indicating ECM degrading enzymes relaxed interstitial collagen and resulted in reduced SS within the tumor interstitium. Provenzano et al. [5] showed that administering PEGPH20 to autochthonous tumors resulted in a four-fold decrease in TTP, 71% increase in discernable vessel >10 μm in diameter, and minimal effect on vessels <10 μm in diameter. Chauhan et al. [10] also applied an angiotensin inhibitor, losartan, which showed a drastic decrease in collagen area fraction and hyaluronic acid area fraction, as well as an increase in perfused vascular area. These results indicate reducing SS should have a significant improvement on functional vascularized area.

The limited correlation between vascular patency and TTP could arise from the difference in measurement techniques. Vascular patency was computed from eight images throughout the interior of the tumor and TTP was a point measurement technique in a single region of the tumor. Additionally, this limited correlation could arise from our choice to exclude the periphery of the tumor from our comparison. The periphery of the tumor is more vascularized than the interior of the tumor and could be used to illustrate a stronger comparison between the two variables. The decision was chosen as such to draw a fair comparison between collagen content and TTP, as both were measured and quantified within the interior of the tumor mass. Further investigation into the relationship between TTP and vascular compression in both the interior and posterior of the tumor will be conducted in a future study in order to clarify the relationship shown in Fig. 4(d).

Our results in Fig. 4(e) illustrated a statistically significant decrease in orthotopic tumor verteporfin uptake in respect to TTP. Due to the molecular size, verteporfin transport is primarily diffusion-weighted, indicating increased TTP, SS, and collagen content play the dominant role in preventing molecular transport into the tumor. Our group has shown that the measurement of TTP is the direct combination of SS and IFP. Our measurements of IFP illustrate a strong convective barrier that could be used to enhance differences between drug uptake and measurements of TTP in the subcutaneous tumor location. Our collaborators have developed photoactive nanoliposomes [40], which depend on convective transport into the tumor interstitium, thereby would be more greatly affected by IFP in drug delivery in both the subcutaneous and orthotopic tumor locations.

In summary, this study introduced a novel pressure measuring technique capable of isolating interstitial free fluid pressure (IFP) and solid stress (SS) from TTP measured using a modified Millar Mikrotip pressure catheter (SPR-671). The results illustrated that TTP is dominated largely by the SS component in these tumors, and that this correlates strongly with interstitial collagen area fraction within pancreatic tumors ($R^2 = 0.799$, $p < 0.001$). The mean values of TTP and IFP were measured to be 48.8 mmHg and 12.8 mmHg, respectively, indicating that solid stress, caused by tensile strength of collagen, results in the elevated TTP measured using a conventional piezoelectric pressure sensor. Global tumor measurements of vascular density (anti-CD31 immunostain) showed a statistically significant increase in vascular density in the orthotopically grown tumor location compared with subcutaneously grown tumors ($p = 0.05$). Vascular perfusion (e.g., verteporfin dosimetry) showed strong correlation with localized measurements of TTP in the orthotopic location ($R^2 = 0.46$, $p < 0.04$) and a statistically significant increase in verteporfin uptake was observed in orthotopic tumor locations compared with subcutaneous tumor locations ($p < 0.01$). This is a pertinent study as our findings illustrate that collagen area fraction directly correlates with TTP, heavily weighted by solid stress, and provides further insight in molecular dosimetry and therapeutic strategy in treating pancreatic cancer.

In a future study, localized measurements of hyaluronic acid, collagen, vascular density, vascular patency, and vascular perfusion will be conducted to more strongly understand complexities that lead to elevated TTP in pancreatic tumors. Administration of collagenase and hyaluronidase will be incorporated to determine the causal role of stromal content on interstitial hypertension. Additionally, convective-transport dependent larger molecules, such as photoactive nanoliposomes [40], will be used to identify a correlation with IFP component of TTP measurements with molecular uptake.

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