Abstract

Purpose: Low–molecular weight (LMW) chemotherapeutics are believed to reach tumors through diffusion across capillary beds as well as membrane transporters. Unexpectedly, the delivery of these agents seems to be augmented by reductions in tumor interstitial fluid pressure, an effect typically associated with high–molecular weight molecules that reach tumors principally through convection. We investigated the hypothesis that improved intratumoral convection can alter tumor metabolism and enhance the delivery of a LMW chemotherapeutic agent to solid tumors.

Experimental Design: For this purpose, we applied 31P/19F magnetic resonance spectroscopy (MRS) and magnetic resonance spectroscopic imaging (MRSI) to examine the influence of type I collagenase on tumor bioenergetics and the delivery of 5-fluorouracil (5FU) to HT29 human colorectal tumors grown s.c. in mice.

Results: Collagenase effected a 34% reduction in tumor interstitial fluid pressure with an attendant disintegration of intratumoral collagen. Neither mice-administered collagenase nor controls receiving PBS showed changes in 31P phosphorus MRS–measured tumor bioenergetics; however, a time–dependent increase in the content of extracellular inorganic phosphate (Pi) was observed in tumors of collagenase-treated animals. 31P phosphorus MRSI showed that this increase underscored a more homogeneous distribution of Pi in tumors of experimental mice. 19F fluorine MRS showed that these changes were associated with a 50% increase in 5FU uptake in tumors of experimental versus control animals; however, this increase resulted in an increase in 5FU catabolites rather than fluoronucleotide intermediates that are required for subsequent cytotoxicity.

Conclusions: These data indicate that the modulation of convective flow within tumors can improve the delivery of (LMW) chemotherapeutics and show the potential role for noninvasive imaging of this process in vivo.

To reach cancer cells, a blood-borne therapeutic agent must move across the vasculature and enter the tumor interstitium. Discouringly, tumors develop phenotypic features that impede drug delivery (1); however, considerable progress has been made toward the delineation of these features (2). The characteristically elevated interstitial fluid pressure observed in solid tumors is an established barrier to the transport of fluid and solute molecules into and within the tumor interstitium (3). An elevated tumor interstitial fluid pressure (TIFP) has been shown to limit significantly convective flow out of capillaries and through the interstitium in accordance with Starling’s hypothesis (4). The prevailing theory suggests that the effect of elevated TIFP in diminishing drug delivery is largely dependent upon the size of the administered therapeutic agent. High–molecular weight (HMW) molecules are transported from the vascular space and through the interstitial space primarily by convection whereas the transport of low–molecular weight (LMW) molecules is driven primarily by diffusion (5, 6). This theory suggests that lowering TIFP, and thereby enhancing transvascular and interstitial convection, can lead to considerable improvements in the delivery of HMW compounds whereas improvements in the delivery of LMW compounds are more likely to derive from increasing the amount entering the tumor microcirculation. Pharmacologic interventions that modulate TIFP have been effective in facilitating the delivery of HMW compounds, including antibodies (7–9) and albumin (10). Unexpectedly, a reduction in TIFP has also been associated with an augmented delivery of LMW compounds, including EDTA (11, 12), camptothecin-11 (13), Taxol (14), epothilone B (15), and 5-fluorouracil (5FU; refs. 14, 16). These results hold important implications for improving the efficacy of other chemotherapeutics and...
Translational Relevance

Inadequate drug delivery is a major cause of drug resistance and methods of enhancing chemotherapy delivery to solid tumors are under investigation. The authors showed, using noninvasive magnetic resonance (MR) imaging techniques, that reductions in tumor interstitial fluid pressure (TIFP) enhances delivery of low–molecular weight drugs. This was unexpected because, typically, high–molecular weight molecules that reach tumors principally through convection are more significantly affected by TIFP. Increased 5-fluorouracil delivery was documented noninvasively by MR spectroscopy but was associated with greater levels of catabolites, affected no changes in anabolites, and had no increased therapeutic benefit. Improved drug delivery may not be adequate for enhancing response, particularly to produgs that need to be activated, which was the case with 5-fluorouracil. Noninvasive methods of imaging changes in TIFP, drug delivery, and drug activation are essential for clinical studies focused on methods of enhancing tumor response. The methods used in this investigation, localized 31P MR and 19F MR studies of global tumor accumulation, have been used in the clinic previously and thus are feasible to apply in future studies aimed at changing TIFP, enhancing drug delivery, and monitoring metabolism of fluoropyrimidine drugs and, possibly, other chemotherapeutic drugs.

emphasize the importance of delineating the mechanism underlying this unexpected augmentation in drug delivery.

The clear exposition of this mechanism is complicated by the intimate relationship between tumor blood flow (TBF) and TIFP. TBF is driven by the pressure difference between the arterial and venous circulation as well as geometric and viscous resistances in tumors (17) and is a primary determinant of the diffusion of LMW molecules in and through tumors. TIFP is principally driven by tumor microvascular pressure, which is associated with arterial and venous pressures through vascular resistance (18). The relationship of TIFP and TBF is underscored by the finding that reductions in TIFP can produce corresponding increases in TBF (19, 20). As a result, decoupling the effects of TIFP and TBF in facilitating drug delivery is a difficult task. In the present study, we applied in vivo nuclear magnetic resonance spectroscopy (MRS) and magnetic resonance spectroscopic imaging (MRSI) to explore the relative contributions of these properties and to test the hypothesis that improved intratumoral convection can enhance the delivery of LMW chemotherapeutic to solid tumors. Toward this goal, we examined the influence of type I collagenase on the delivery of 5FU to HT29 human colorectal tumors grown s.c. in mice. TIFP was measured with the use of a transducer-tipped catheter and modulated through the systemic administration of type I collagenase, an agent known to lower TIFP and increase the uptake of monoclonal antibodies (8). 31Phosphorus (31P)MRS-detected tumor bioenergetics were used as a surrogate of TBF whereas 31P MRSI was applied to assess spatial variations in the distribution of phosphorus metabolites. 5FU pharmacokinetics were monitored by 19Flourine (19F)MRS to measure associated changes in drug delivery. These studies emphasize the role of noninvasive imaging in characterizing and optimizing the modulation of the tumor microenvironment for improved drug delivery.

Materials and Methods

Cell line

The HT29 human colon adenocarcinoma cell line was obtained from American Type Culture Collection. The cell line was grown in McCoy’s 5A medium (modified) with 1.5 mmol/L l-glutamine adjusted to contain 2.2 g/L sodium bicarbonate and 10% FCS.

Mouse model

Tumors were established through the s.c. injection of 3 million HT29 cells into the left lower extremity of 5-to-7-wk-old male nude mice (nu/nu; Charles River Laboratories). Tumor dimensions were measured by caliper, and tumor volumes were determined according to the ellipsoid volume formula (V = 6 × (width)2 × length). Tumors reaching a volume of 400 ± 75 mm3 were selected for experiment. Animal studies were conducted according to institutionally approved protocols for the safe and humane treatment of animals.

Interstitial fluid pressure measurements

A Mikro-Tip catheter pressure transducer (model SPC 320; Millar Instruments) was used to measure the interstitial fluid pressure at the center of each tumor as described previously (21). The pressure transducer was connected to a bridge amplifier (model ML110; AD Instruments), and the amplified signal was transferred to a four-channel data acquisition system (model ML845; AD Instruments). Before each measurement, an 8-point calibration was done with a digital manometer. For the duration of the experiment, each animal was maintained under isoflurane anesthesia (1.5%) at 37°C with the use of an isothermal pad. Isoflurane anesthesia was selected based on its ability to maintain stable cardiovascular parameters, including cardiac output and TBF, to limit the potential influence of anesthetic-related vascular reactivity on interstitial fluid pressure measurements (22, 23). Isoflurane is known to affect a relatively modest decline in mean arterial blood pressure; however, an isolated reduction in mean arterial blood pressure has not been found to influence TIFP (24). The distance from the skin to the center of the tumor was estimated and used to mark the catheter to ensure accurate positioning of the transducer. The transducer was introduced into the tumor through an 18-gauge needle. Once the bevel was submerged in the tumor, the catheter was advanced to the center of the tumor and the needle was removed. After a stable interstitial fluid pressure value was achieved for 10 min, 100 µL of PBS or 100 µL of 0.1% collagenase (Clostridiopeptidase A; Sigma) was injected i.v. via a previously placed tail-vein catheter. The TIFP was recorded at 40 Hz for 60 or 120 min after injection.

MR spectroscopy and imaging

MR studies were done on a Bruker Biospec 70/30 small-animal imaging system operating at 7 Tesla and equipped with a 200 mT/m gradient coil insert (Bruker). Experiments were conducted with the use of a temperature-control-susceptibility-matching water bath (25). Spectral data were analyzed with the use of the jMRUI software package (26). Quantitation of spins was achieved through the use of the AMARES nonlinear least squares quantitation algorithm (27). Spectroscopic images and parametric maps were generated with the SITOOLS package and Paravision software (Bruker; ref. 28).

31Phosphorus MRS. Each mouse was briefly anesthetized with the use of methoxyflurane (Medical Developments International, Ltd.). The animal was then positioned within a Faraday shield, and the tumor-bearing or non–tumor-bearing leg was extended through a custom-built, 4-turn 31P solenoidal coil of 10 or 11 mm inner diameter. Tumor immobilization was achieved by securing the foot between two fixed foam pads. The mouse was allowed to regain consciousness and was...
conscious for the remainder of the experiment. The shield and coil were immersed in the water bath and positioned at the isocenter of the magnet. Subsequent shimming optimized the magnetic field homogeneity to a proton line width of ∼30 to 60 Hz. In vivo pulse angles were then calibrated with the use of signals derived from endogenous phosphorus metabolites. The animal was removed from the magnet, and a 100-μL injection of 0.1% collagenase (n = 3) or PBS (n = 5; n = 3) was administered i.v. to tumor-bearing or non–tumor-bearing animals, respectively, without removing the mouse from the probe. The animal was then remounted in the probe holder, and the coil was replaced at the isocenter of the magnet. Optimized acquisition parameters included a 45° pulse angle, a 0.5-s pulse repetition time, and a spectral width of 20 kHz in 2,048 data points. Serial spectra were acquired as the average of 1,200 excitations, enabling a temporal resolution of 10 min per spectrum. For purposes of quantitation, a microsphere containing 18 μL of a 75-mmol/L methylene diphosphonic acid (Sigma-Aldrich Corp.) aqueous solution was positioned adjacent to the coil. A single, fully relaxed spectrum of this reference was acquired to ensure accuracy.

31Phosphorus MRSI. Each mouse was prepared for the experiment as described above. After the calibration of pulse angles, the animal was removed from the magnet and a 100-μL injection of 0.1% collagenase (n = 3) was administered i.v. The mouse was then remounted in the coil holder, and the coil was replaced at the isocenter of the magnet. In vivo 31PMRS images were acquired with the use of custom-built, 4-turn 31P solenoidal coils of 10, 11, or 12 mm inner diameter. A weighted acquisition scheme was applied to acquire two-dimensional images with 13 phase-encode steps and an in-plane resolution of 2.0 × 2.0 mm. The total imaging time was 2 hr. 31PMRSI spectral parameters included a 45° pulse angle, a 0.5-s pulse repetition time, and a spectral width of 20 kHz in 2,048 data points. For purposes of quantitation, a microsphere containing 18 μL of a 75-mmol/L methylene diphosphonic acid (Sigma-Aldrich Corp.) aqueous solution was positioned adjacent to the coil. A single, fully relaxed spectrum of this reference was acquired to ensure accuracy. Before 31PMRSI, a proton image was acquired at 300 MHz for anatomical localization of the MRSI data. A Rapid Acquisition with Relaxation Enhancement (RARE) sequence was used with 40 mm field of view, 192 × 256 matrix size, 6 signal averages, RARE factor of 8, 1,000 ms repetition time, and 102 ms echo time.

19Fluorine MRS. Each mouse was briefly anesthetized with the use of methoxyflurane and allowed to regain consciousness 2 hr before positioning to facilitate the calibration of in vivo pulse angles as described previously (29). One hour later, a 100-μL injection of 0.1% collagenase (n = 5) or PBS (n = 5) was administered i.v. At this time, the mouse was anesthetized again and prepared for the experiment as described above. A copper Faraday shield was used in place of the 60-cm syringe barrel. The use of a copper Faraday shield allowed for the in vivo calibration of pulse angles by suppressing fluorescent signals arising from within the body (30). In vivo pulse angles were calibrated from the methoxyfluorouracil resonance. The animal was removed from the magnet and a 300-μL injection of SFU (150 mg/kg Adrucil; Pfizer) was administered i.v. (−110 min after administration of collagenase or PBS). The mouse was then remounted in the coil holder, and the coil was replaced at the isocenter of the magnet. In vivo 19F magnetic resonance (MR) spectra were acquired with the use of a custom-built, 2-turn 19F solenoidal coil of 10.7 mm inner diameter. Optimized acquisition parameters included a 60° pulse angle, a 1.7-s pulse repetition time, and a spectral width of 40 kHz in 1,024 data points. Serial spectra were acquired as the average of 355 excitations, enabling a temporal resolution of 10 min per spectrum. For purposes of quantitation, a microsphere containing 18 μL of a 150-mmol/L sodium fluoride (Sigma-Aldrich Corp.) aqueous solution doped with 15 mmol/L Magnesist (Berlex Laboratories Inc.) was positioned adjacent to the coil. A single, fully relaxed spectrum of this reference was acquired to ensure accuracy.

Tumor growth delay

Tumor width (minor diameter) and length (major diameter) were measured by caliper. Tumor volumes were calculated according to the formula for an ellipsoid, \( V = \pi/6 \times (width)^2 \times length \). Twenty-five animals were studied as four cohorts: untreated control mice (n = 10), mice injected with 0.1% collagenase i.v. (n = 5), mice injected with PBS + 150 mg/kg SFU i.v. (n = 5), and mice injected with 0.1% collagenase + 150 mg/kg SFU i.v. (n = 5). SFU was administered −110 minutes after PBS or collagenase injection. Gompertzian analysis was applied for the assessment of tumor growth characteristics as described previously (31).

Immunohistochemistry

The immunohistochemical staining was done by a Discovery XT System (Ventana Medical Systems). Detection of type I collagen in paraffin-embedded tumor sections involved a 32-min period of blocking using 10% normal rabbit serum and 2% bovine serum albumin. Incubation with the polyclonal goat anti-collagen type I primary antibody (0.5 μg/mL; Biodesign) was carried out for 3 hr at room temperature followed by a 20-min incubation with biotinylated rabbit anti-goat secondary antibody (Vector Labs) at a dilution of 1:200. A 3,3-diaminobenzidine detection kit containing blocker D, Diaminobenzidine, and streptavidin–peroxidase D, and 3,3-diaminobenzidine D was used according to manufacturer instructions (Ventana Medical Systems).

The Metamorph Imaging System (Molecular Devices Corporation) was utilized for the display and analysis of tissue sections. Collagen content of stained tissue sections was determined as the percentage of horseradish peroxidase–positive pixels representing type I collagen. Before quantitation, background signals were removed. Subsequently, pixels in the type I collagen (brown) range were selected by signal thresholds, and regions of interest analysis was done to quantitate “positive” and “negative” pixels. Three separate regions of interest were analyzed from each tissue. Sectioned tumors were collected 2 hr after injection of 0.1% collagenase (n = 3) or PBS (n = 3).

Statistical analysis

Statistical analyses were done with the use of GraphPad Prism version 4.03 for Windows (GraphPad Software). Mean values are reported as mean ± SE of the mean. The two-tailed unpaired t test was done to assess the statistical significance of differences in mean values.

Results

Collagenase effectively reduces TIFP. The systemic administration of 0.1% collagenase effected a consistent and characteristic reduction in the interstitial fluid pressure of s.c. tumors (Fig. 1A). TIFP reached a mean nadir of 64% ± 2.7% of its initial value at 120 minutes after injection. The time course of collagenase-induced TIFP modulation was marked by an initial and immediate increase, associated with a presumed increase in intravascular volume associated with the injection, followed by a phase of rapid decay and a subsequent phase of slower decline. The rapid phase of decay was characterized by a decrease in TIFP to 75% of its initial value within 40 minutes of collagenase administration. The elicited reduction in TIFP was found to be highly significant when compared with PBS-injected controls (\( P = 0.003 \)).

Immunohistochemical staining of tissue sections for type I collagen revealed a substantial difference in the amount of type I collagen in the tumors of PBS and collagenase-treated mice (Fig. 1B, C). Type I collagen staining accounted for 24.1% ± 1.3% of the pixels in tumor sections from PBS-treated animals. These sections showed pronounced collagen staining at the
periphery and heterogeneous collagen staining within the tumor (Fig. 1B). This pattern of collagen staining was duplicated in tumors from collagenase-treated animals, with the exception of an attenuation in the density of collagen found both at the periphery of and within the tumors (Fig. 1C). This attenuation was reflected in a reduced percentage of type I collagen–positive pixels in these sections as compared with tumor sections from PBS-treated animals (14.0% ± 2.0%; \( P = 0.01 \)). The influence of the reduced collagen content observed in tumors of collagenase-treated animals was further emphasized by the compromised integrity of these tissue sections. Compared with tumors of PBS-treated animals, the tissue within tumors of collagenase-treated animals was loosely connected and often discontinuous (Fig. 1B, C).

**Fig. 1.** Type I collagenase effects a marked reduction in TIFP associated with a corresponding reduction in intratumoral collagen. A, TIFP relative to the time of PBS (C) or 0.1% collagenase (D) injection. TIFP values are reported at 10-min intervals and are normalized to the pressure assessed 10 min before injection. A transient increase in TIFP was observed immediately after injection, presumably associated with the increase in vascular volume. B and C, composition of representative tumor sections from PBS-treated (B) or collagenase-treated (C) mice stained for type I collagen.

**Fig. 2.** Collagenase does not significantly alter tumor bioenergetics but effects a significant increase in extracellular Pi. \(^{31}\)PMR spectroscopic evolution of phosphorus metabolites in the tumor-bearing legs of mice administered PBS (A) or 0.1% collagenase (B). Serial \(^{31}\)PMR spectra from representative animals are shown. The start of each spectral acquisition relative to the time of injection is indicated. Inset, resolved metabolite peaks, including (1) phosphoethanolamine, (2) phosphocholine, (3) PCr, (4) P\(\text{Im}^\text{major} \), (5) phosphocreatine, (6) \(\gamma\)-NTP, (7) \(\alpha\)-NTP, (8) NAD\(^+\), (9) diphosphodiesters, and (10) \(\beta\)-NTP.
Collagenase does not alter tumor bioenergetics. After the systemic administration of 0.1% collagenase or PBS, tumor phosphorus metabolites were serially monitored by nonlocalized $^{31}$PMRS over the course of 4 hours (Fig. 2A and B, respectively). The use of a susceptibility-matching water bath enabled the clear resolution of individual phosphomonoesters as well as two species of inorganic phosphate (Pi) designated $P_{\text{major}}$ and $P_{\text{minor}}$. Based on the observed chemical shifts of these Pi peaks, the more acidic $P_{\text{minor}}$ has been attributed to the extracellular fraction of Pi whereas $P_{\text{major}}$ was identified as Pi within tumor cells (32). Unexpectedly, a minimal change in the levels of the resolved phosphorus metabolites was appreciated in the tumors of either PBS-treated or collagenase-treated animals. As a result, the tumor bioenergetic status, as assessed by the ratio of total nucleoside triphosphates [$NTP$ ($\alpha$-NTP, $\beta$-NTP, $\gamma$-NTP)] to intracellular Pi ($NTP/P_{\text{major}}$), seemed to be stable for both cohorts of mice (Fig. 3A). Interestingly, a time-dependent increase in the level of extracellular Pi was observed in the tumors of collagenase-treated mice but was not appreciated in the tumors of PBS-treated animals. Approximately 70 to 80 minutes following the i.v. administration of collagenase, the levels of extracellular Pi began to increase, accumulating to an average of 1.6 times their initial value (Fig. 3B). When compared with the time course evolution of extracellular Pi in control tumors, this increase was found to be statistically significant ($P = 0.001$). This increase was not associated with a change in extracellular pH as indicated by the stability of the chemical shift for the $P_{\text{minor}}$ resonance over 4 hours. $^{31}$PMRS experiments were done on non-tumor bearing legs of mice to assess differences in phosphorus metabolites between PBS-treated and collagenase-treated animals. These experiments revealed spectra consistent with muscle tissue and showed no changes in the levels of resolved phosphorus metabolites for collagenase-treated or PBS-treated mice (data not shown).

Two-dimensional $^{31}$PMR spectroscopic images were collected to assess the spatial distribution of phosphorus metabolites in the tumors of mice treated with PBS or collagenase (Fig. 4A and B). The images were acquired at a high spatial resolution (in-plane resolution of $2.0 \times 2.0$ mm) to enable the evaluation of several tumor regions. Consistent with the nonlocalized spectral data, similar distributions of phosphorus metabolites reflecting equivalent tumor bioenergetic profiles were discerned for PBS-treated and collagenase-treated mice. Parametric mapping revealed that the increased levels of extracellular Pi noted in the tumors of collagenase-treated versus PBS-treated mice were associated with an improved distribution of this metabolite within tumors as compared with those of PBS-treated mice (Fig. 4A and B, respectively).

Collagenase improves the delivery of 5FU. To study the influence of collagenase on the delivery of LMW therapeutics, 5FU pharmacokinetics were serially monitored by $^{19}$FMRS. Four fluorinated metabolites were distinguished and identified based on observed chemical shifts, including 5FU, fluoronucleotides (designated as $N$Ucs, which include fluorouridine monophosphate, fluorouridine diphosphate, fluorouridine triphosphate, fluoroerythropurine monophosphate, fluoroerythropurine diphosphate, and fluoroerythropurine triphosphate), $\alpha$-fluoro-$\beta$-ureidopropionic acid (FUPA), and $\alpha$-fluoro-$\beta$-alanine (33). Collagenase substantially enhanced the delivery of 5FU to the tumor (Fig. 5A). $^{19}$FMRS-measured levels of 5FU in collagenase-treated mice were elevated to an average of 1.5 times those found in PBS-treated animals ($P = 0.0185$). This finding was underscored by the presence of detectable 5FU concentrations at an extended time point (48 minutes post-injection) in collagenase-treated mice. Linear regressions of the fitted $^{19}$FMRS time course data revealed a statistically insignificant increase in the elimination rate of 5FU in collagenase-treated versus PBS-treated animals ($y = 4.92-0.086x$ and $y = 3.99-0.081x$, respectively). The significantly increased 5FU concentrations did not translate into higher levels of the anabolic fluoronucleotides that are essential precursors for cytotoxicity; $N$Ucs accumulated to similar levels and with analogous kinetics in the experimental and control groups (Fig. 5B). Interestingly, however, significantly higher quantities of the 5FU catabolite FUPA were measured in tumors of collagenase-treated animals ($y = 4.92-0.086x$ and $y = 3.99-0.081x$, respectively). The significantly increased 5FU concentrations did not translate into higher levels of the anabolic fluoronucleotides that are essential precursors for cytotoxicity; $N$Ucs accumulated to similar levels and with analogous kinetics in the experimental and control groups (Fig. 5B). Interestingly, however, significantly higher quantities of the 5FU catabolite FUPA were measured in tumors of collagenase-treated animals ($y = 4.92-0.086x$ and $y = 3.99-0.081x$, respectively). The significantly increased 5FU concentrations did not translate into higher levels of the anabolic fluoronucleotides that are essential precursors for cytotoxicity; $N$Ucs accumulated to similar levels and with analogous kinetics in the experimental and control groups (Fig. 5B). Interestingly, however, significantly higher quantities of the 5FU catabolite FUPA were measured in tumors of collagenase-treated animals ($y = 4.92-0.086x$ and $y = 3.99-0.081x$, respectively).
discernible levels of catabolites were not appreciated (data not shown).

**Collagenase does not influence efficacy of 5FU.** 5FU caused a significant growth delay in HT29 tumors as compared with untreated tumors (Fig. 6; *P* = 0.001); however, consistent with the 19F MRS data showing similar levels of FNucs in both the control and experimental groups, tumor growth delay studies failed to show a significant difference in the tumor growth rates of mice treated with collagenase + 5FU or PBS + 5FU (Fig. 6). Gompertzian analysis determined a specific growth rate of 0.039 day⁻¹ for tumors in mice treated with collagenase and 5FU, corresponding to a tumor doubling time of 5.4 days. The specific growth rate for tumors in mice treated with PBS and 5FU was found to be 0.039 day⁻¹, with an associated doubling time of 5.5 days. Similarly, tumors in mice treated with collagenase showed a growth rate (0.071 day⁻¹) and doubling time (3.0 days) comparable with those in untreated mice (0.065 day⁻¹; 3.3 days).

**Discussion**

The manipulation of barriers to drug delivery in solid tumors holds great promise for the optimization of cancer therapy. The realization of this potential requires that the influence of each of these barriers on our current therapeutic arsenal be explicitly characterized. The present study addresses this need and introduces specific evidence that the modulation of convective currents within tumors enhances the delivery of a conventional LMW chemotherapeutic. This insight, achieved through the use of noninvasive spectroscopy and imaging strategies, contradicts current theory suggesting a limited role for convection in the transport of LMW therapeutics across the microvasculature and through the tumor interstitium.

The systemic administration of collagenase induced a significant reduction in TIFP that was associated with the degradation of interstitial type I collagen. These data support the findings of a previous study reporting similar results after the systemic administration of collagenase (8). Eikenes et al. observed reductions in both TIFP and microvascular pressure and attributed the latter to a reduction in vascular resistance caused by the specific degradation of vessel-associated collagen. This decline in microvascular pressure offers significant insight into the kinetics of the observed modulation in TIFP. As the primary force governing TIFP, the reduced microvascular pressure is the expected source of the fast initial drop in TIFP (8, 18). The enzymatic decomposition of interstitial collagen and consequent increase in hydraulic conductivity explains the measured slow phase of TIFP decay (34). These changes result in a collagenase-mediated restoration of the transcapillary pressure gradient that has been shown to enhance convective flow within tumors and to thereby facilitate the delivery of a HMW therapeutic agent (8). The data described here as well as in the literature (8) show that collagenase provides an effective and consistent means of modulating TIFP. Synthesized primarily by tumor-associated host fibroblasts, type I collagen is a major constituent of the tumor extracellular matrix and plays a central role in impeding solute transport within the tumor interstitium (35). Although the collagen does not represent the most abundant component of the tumor extracellular matrix, the total tissue collagen content was found to correlate more significantly with the functional...
properties of tumors that influence the diffusion coefficient of solutes, including tissue elastic modulus and hydraulic conductivity. Nevertheless, the demonstrated role of collagenase in facilitating the metastatic cascade limits the potential for the clinical application of this agent (36); however, the findings presented in this study underscore the importance of developing agents to modulate TIFP.

The fundamental relationship between TIFP and TBF dictates that changes effected in one can often influence alterations in the other. A preponderance of studies investigating the therapeutic adjustment of TIFP has reported associated alterations in TBF (24, 37–41). Moreover, the manipulation of TBF has been applied to modulate TIFP (42). The present study applied 31PMRS and 31PMRIs to measure tumor bioenergetics as a surrogate index of TBF. Although not a direct measure of blood flow, energy metabolism is strongly coupled to blood flow, and 31PMR spectra are therefore very sensitive to changes in blood flow. Previous studies have shown that the modulation of TBF through radiation, chemotherapy, as well as thermal therapy is associated with rapid and predictable changes in the NTP/Pi ratio (43–46). This relationship is underscored by the robust and congruent changes in TBF and NTP/Pi ratio associated with administration of vasoactive agents (47, 48). 31PMRS revealed a constant tumor NTP/Pi ratio after collagenase treatment similar to that observed in the tumors of mice treated with PBS. 31PMRIs confirmed this finding showing similar bioenergetic profiles throughout tumors of mice treated with collagenase or PBS. The limited effect of collagenase on TBF was further suggested by the absence of any change in extracellular pH over the monitored time course. As the primary modulator of substrate supply, TBF is largely responsible for changes in the tumor microenvironment that result from tumor metabolism (49). Thus, despite lowering microvascular pressure, collagenase does not seem to alter TBF.

In the absence of altered TBF, the unexpected kinetic and dynamic patterns of extracellular Pi in tumors of collagenase-treated mice are likely a consequence of enhanced convective flow. The renewed pressure gradients associated with the decreased TIFP could generate a swirling flow or percolation of fluid across the capillary membrane and through the tumor interstitium, carrying blood-borne solutes with it. The expected result is an expansion in the level and distribution of these solutes within the tumor. Consistent with this effect, the maximal increase in extracellular Pi corresponds to the nadir in TIFP. Moreover, spectroscopic imaging revealed a more uniform distribution of extracellular Pi within tumors of mice treated with collagenase. Taken together, these data indicate that collagenase modulates the tumor microenvironment by lowering TIFP and improving convective currents without affecting TBF.

**Fig. 5.** Collagenase increases intratumoral concentrations of 5FU. 19FlMRS-determined 5FU pharmacokinetics in tumors of experimental or control mice. Spectra were acquired with a 10-min temporal resolution. The fitted data were normalized to an external reference standard and reported as the normalized signal amplitude (AUC). A, serial measurements of mean 5FU levels show its accumulation to significantly higher levels in tumors of collagenase-treated mice (▲) as compared with PBS-treated controls (●; *, ***, P = 0.0185). Linear regressions of these time-course data are shown for both groups (y = 4.92 - 0.086 x; r² = 0.993 and y = 3.99 - 0.081 x; r² = 0.991, respectively). B, serial measurements of mean FNuc levels in tumors of collagenase-treated (▲) and PBS-treated (●) mice. C, serial measurements of mean FUPA levels in tumors of collagenase-treated (▲) and PBS-treated (●) mice.

**Fig. 6.** Collagenase does not influence the growth delay of tumors treated with 5FU relative to 5FU-treated controls. Mean tumor growth curves for s.c. HT29 tumors growing in the legs of control (untreated; △) mice or mice receiving collagenase only (●), PBS + 5FU (▲) or collagenase + 5FU (■). The data are presented as transformed Gompertz tumor volumes plotted against time. The best-fit linear regression lines with slope α for these data are indicated, where α is the specific growth rate (y = 1.22 - 0.07x, r² = 0.974; y = 1.30 - 0.07x, r² = 0.982; y = 1.27 - 0.03x, r² = 0.962; and y = 1.25 - 0.03x, r² = 0.975, respectively).
The $^{31}$PMRS results presaged the finding that an improvement in intratumoral convective flow specifically enhances the delivery of a LMW chemotherapeutic. Although a collagenase-mediated facilitation of 5FU diffusion cannot be completely excluded, Magzoub et al. have shown that the influence of type 1 collagenase on the translational diffusion of exogenous compounds through s.c. tumors is size dependent and likely to be negligible for LMW agents (35). This observation has great relevance to clinical cancer therapy because inadequate delivery of conventional chemotherapeutics is a well-known limitation to the efficacy of current regimens (50). 5FU, a first-line chemotherapeutic agent in the treatment of colorectal cancer, has a response rate of only 15% to 20% (51). Increasing the amount of 5FU that is delivered to susceptible tumors might improve these rates. The marked first-pass effect associated with systemic 5FU therapy further emphasizes the importance of maximizing the levels of this agent that can access the targeted tumor with each round of circulation. Given the presence of interstitial hypertension in solid tumors of cancer patients, our data suggest that the modulation of TIFP might be applied to improve the delivery and efficacy of LMW chemotherapeutics in the clinic (52–54).

Upon reaching the tumor interstitium, the initial barrier to 5FU activity involves the transport of the prodrug across tumor cell membranes into the intracellular space. This process is governed by active transport that is catalyzed by the uracil transporter system and driven by the extra-to-intracellular pH gradient (55, 56). This process has been reported to be responsive to temperature, intracellular ATP concentration, extracellular sodium concentration, as well as the extracellular concentration of 5FU (57–59). The dominant feature of the measured 5FU pharmacokinetic profiles was an increase in 5FU concentration within the tumors of collagenase-treated animals. The detectability of 5FU to an additional time point in the spectra of collagenase-treated as compared with PBS-treated animals is likely a consequence of the higher initial levels of 5FU in combination with similar rates of 5FU elimination for these two groups. Because 5FU is a prodrug, increasing the quantity of this agent reaching the intracellular compartment is not necessarily sufficient to ensure optimized therapy (60). The efficacy of 5FU therapy is dependent upon the intracellular conversion of the administered prodrug to FNucs. Because the rate of 5FU transport is rapid at low concentrations relative to the rate of 5FU activation to fluoronucleotides (56), it is possible that the latter step limits the efficient use of the augmented intratumoral 5FU concentrations. Suboptimal anabolism of the 5FU prodrug might also explain the absence of an enhanced tumor growth delay for collagenase + 5FU–treated animals; however, the finding that the transport of 5FU in Ehrlich cells obeys a hyperbolic saturation curve and is not concentration dependent at higher concentrations suggests the possibility of an alternative mechanism (57) in which intracellular transport is the limiting factor to enhanced 5FU efficacy.

Despite the demonstrated enhancement in 5FU uptake for tumors of mice treated with collagenase, there was no improvement in response. $^{19}$FMRS-monitored 5FU pharmacokinetics revealed elevated levels of fluorinated catabolites but no change in MR–detectable fluorinated anabolites in tumors of mice treated with collagenase + 5FU. The increased catabolism of 5FU suggests that the interplay of the anabolic and catabolic enzymes responsible for cellular metabolism of 5FU was responsible for the absence of an extended growth delay for tumors of mice treated with collagenase + 5FU. It is possible that the 5FU levels achieved in both cohorts saturated the anabolic enzymes, thereby favoring 5FU catabolism. The relative role of 5FU metabolism in determining therapeutic efficacy is likely to be tumor dependent because it has previously been shown that increased levels of 5FU within tumors can affect improved tumor growth delays (16). Whereas the benefit of modulating 5FU delivery is clear, the presented data underscore the importance of modulating the levels and activity of enzymes involved in both 5FU anabolism and catabolism. Inhibitors of dihydropyrimidine dehydrogenase have been shown to dramatically enhance response rates to 5FU, and gene therapy strategies designed to increase levels of anabolic enzymes show considerable promise (61, 62).

In the context of existing preclinical data clearly indicating that the modulation of interstitial hypertension can potentiate drug delivery (4), the broader application of this strategy necessitates a means of noninvasively mapping changes in TIFP. Initial studies into the use of MR imaging for this purpose examined the correlation of proton relaxation times with interstitial fluid pressure levels (63). Recently, Hassid et al. described contrast-enhanced MR imaging that measured the influence of TIFP on the distribution of a gadolinium-based contrast agent (64). This approach produced quantitative maps of regional contrast-agent levels that correlated well with measured TIFP. The data presented in the current study suggest that $^{31}$PMRSI might offer an alternative means of visualizing regional variations in TIFP that would have the advantage of imaging an endogenous species while simultaneously providing information about tumor bioenergetics and phospholipid metabolism. Furthermore, phosphorus MRSI studies of human cancers may be useful in the assessment of disease prognosis (65). The parallel application of diffusion spectroscopy, a tool for measuring molecular diffusion, could provide quantitative insights into regional pressure gradients (66).

In summary, the extensive use of MRS allowed the detailed study of the role of convective flow in enhancing the delivery of 5FU chemotherapy. $^{31}$PMRS facilitated extended time-course measurements of tumor bioenergetics in each tumor, providing important information on TBF. $^{19}$FMRS enabled the measurement of 5FU pharmacokinetics required for the accurate evaluation of prodrug delivery. Finally, the use of $^{31}$PMRSI in the regional assessment of intratumoral phosphate distribution raises the potential for clinically relevant, noninvasive measurement of TIFP modulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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