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ORIGINAL ARTICLE

Ultrasound-guided intratumoral administration of collagenase-2 improved liposome drug accumulation in solid tumor xenografts

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Abstract
Purpose To investigate the effect of intratumoral administration of collagenase-2 on liposomal drug accumulation and diffusion in solid tumor xenografts.
Methods Correlation between tumor interstitial fluid pressure (IFP) and tumor physiological properties (size and vessel fraction by B-mode and Doppler ultrasound, respectively) was determined. IFP response to intravenous or intratumoral collagenase-2 (0.1%) treatment was compared with intratumoral deactivated collagenase-2. To evaluate drug accumulation and diffusion, technetium-99 m-(^{99m}Tc)-liposomal doxorubicin (Doxil^{TM}) was intravenously injected after collagenase-2 (0.1 and 0.5%, respectively) treatment, and planar scintigraphic images acquired and percentage of the injected dose per gram tissue calculated. Subsequently, tumors were subjected to autoradiography and histopathology.
Results IFP in two-week-old head and neck squamous cell carcinoma xenografts was 18 ± 3.7 mmHg and not correlated to the tumor size but had reverse correlation with the vessel fraction ($r = -0.91$, $P < 0.01$). Intravenous and intratumoral collagenase-2 use reduced IFP by a maximum of 35–40%. Compared to the control, the low IFP level achieved through intratumoral route remained for a long period (24 vs. 2 h, $P < 0.05$). SPECT images and autoradiography showed significantly higher ^{99m}Tc-Doxil^{TM} accumulation in tumors with intratumoral collagenase-2 treatment, confirmed by %ID/g in tumors ($P < 0.05$), and pathological findings showed extensive distribution of Doxil^{TM} in tumors.
Conclusions Intratumoral injection of collagenase-2 could effectively reduce IFP in HNSCC xenografts for a longer period than using intravenous approach, which allowed for more efficient accumulation and homogeneous diffusion of the Doxil^{TM} within the tumor interstitium.

Keywords Solid tumor · Interstitial fluid pressure · Collagenase · Drug delivery · Liposome

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Introduction

The success of drug-based tumor treatment involves various factors including dose, administration route, drug accumulation and penetration within the tumor, and the uptake by tumor cells. Optimization of these factors could improve therapeutic outcomes. Pathophysiologically, tumors comprise cancer cells, nutrient vessels, host stromal cells and the supporting extracellular matrix. With tumor progression, an elevation of interstitial fluid pressure (IFP) in solid tumors has been reported [1].

The underlying mechanism of IFP elevation has been investigated and attributed to a series of abnormal changes from parenchymal to stromal components [2, 3]. With careful scrutinization, these intertwined abnormalities might be categorized into two groups: (1) initiating factors, including the highly permeable vascular vessels, dysfunctional lymphatic vessels, and proliferating tumor cells and (2) maintaining factors, including disorderly extracellular matrix and tumor capsulation. Initiating factors can result in the escape of excessive plasma contents into the interstitial space. When normal exchange mechanism between intravascular and interstitial spaces exists, a majority of these exudates are reabsorbed and the interstitial space maintains a relatively constant volume, so the interstitial space has transient IFP elevation [4]. However, the changed matrix interrupts this dynamic fluid exchange and hampers the clearance of the exudate from the interstitial space, which leads to the continuous buildup of IFP and inhibits the convection flux that is deemed as the driving force for the extravasation of entities with size of 10 kDa and above, such as liposomes [5]. As the major component of solid tumor extracellular matrix, collagen has been proven to be the determinant factor for tumor mechanical stiffness, which correlates with the poor penetration of macromolecules [6]. Several studies have suggested that modulating collagen can improve the access and uptake of antibodies in the tumor by "loosening" the tumor interstitium and lowering the IFP. A peptide hormone, relaxin, secreted in the ovary and placenta during pregnancy with the capability of remodeling collagen by inhibiting collagen synthesis, increasing collagen turnover, and up-regulating matrix metalloproteinases has been proven effective in enhancing the diffusion of anticancer drugs in soft tissue sarcomas [7, 8].

The use of collagenases for modulation of tumor matrix has been proven useful in reducing the interstitial pressure in animal models [9, 10]. However, problems ensuing from this procedure block its translation from the laboratory to the clinical setting. The systematic administration of collagenases may cause adverse effects to other healthy tissues and organs. Additionally, the probability of the increasing metastatic risk has to be taken into serious consideration and thoroughly investigated. To the best of our best knowledge, collagenases used in these previous studies were either collagenase-1 or crude mixture of collagenase-1, collagenase-2, and collagenase-3, delivered via an intravenous route.

Secreted by neutrophils and endothelial cells, collagenase-2 initiates cleavage of type I collagens and has little if any effect on the degradation of type IV collagens. A clinical study demonstrated expression of collagenase-2 in HNSCC was extremely low, or nil, which indicated that the role of this collagenase in HNSCC growth is less crucial than the others [11]. Although collagenase-2 may have a potential role in the proteolysis of connective tissue associated with the spread of invasive squamous cell carcinoma as well as ovarian cancer [12], results from various reports implicated collagenase-2 may have a dual role in the tumor progression, when taking into consideration the finding that collagenase-2-deficient mice suffered an increasing incidence of chemically induced skin carcinomas [13].

Clinically, regarding the management of solid tumors, ultrasound-, computed tomography (CT)-, or magnetic resonance imaging (MRI)-guided therapy has been widely accepted as a critical procedure for precise administration of therapeutic agents and dynamic evaluation of therapeutic outcomes. The necessary procedure to generate pathohistological diagnosis before initiation of appropriate therapy. In this study, we hypothesized that ultrasound-guided intratumoral administration of purified collagenase-2 would be effective in reducing interstitial pressure by modulating extracellular matrix due to directly increasing the local collagenase concentration in tumor and subsequently facilitating drug diffusion and accumulation compared with an intravenous route, thereby improving the therapeutic efficacy. To validate the hypothesis, a nude rat HNSCC xenograft model was used to establish the correlation between IFP and other tumor parameters with tumor growth (tumor volume and vessel fraction determined by B-mode and Doppler ultrasound, respectively). Then modulation of collagenase-2 on IFP and the effect on the availability of liposome-encapsulated doxorubicin (Doxil^{TM}) to the tumor and its distribution within the tumor were determined by scintigraphic imaging, autoradiography, and histopathology.

Materials and methods

Cell culture and tumor xenograft

SCC-4 cells (ATCC, Manassas, VA) at 80 ~ 90% confluence were trypsinized and made single cell suspension by aspirating. The cell pellets of 5×10^6 cells were resuspended in 0.2 ml saline (Abbott Laboratories, Abbott Park, IL) in tuberculin syringes for injections. Four-week-old athymic nude rats (rnu/rnu, female) were purchased from Harlan (Indianapolis, IN) and housed in the Laboratory Animal Resource central facility. Procedures in handling animals were in accordance with the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. After anesthesia with 2–3% isoflurane (Abbott) in medical air, the rats were placed in a prone position, and the cell suspension was injected slowly into the lateral subcutaneous compartment (left and right) at the posterior area of the neck to produce the two-tumor xenograft model.

Interstitial fluid pressure measurement

A Samba fiber-optic pressure recording (FOPR) system (Samba Sensors AB, Sweden) was applied. For each tumor, IFP was measured from two sections, one perpendicular to the body (sagittal or coronal) and the other parallel to the body (transverse). In each section, three IFP measurements were recorded, one located in the tumor center and the other two at half way between the center and the periphery at either side, using ultrasound guidance for needle placement with the Vevo 770 microultrasound system (VisualSonics, Toronto, Canada) (Fig. 1a). A 21-gauge needle was used to introduce the measuring fiber into tumors with the FOPR tip being located at the needle orifice. After reaching the indicated measuring point, the needle was withdrawn 3–5 mm to allow the FOPR tip to be fully exposed to tumor interstitial environment. The stable reading was recorded as the IFP at that point.

Collagenase-2 application

Collagenase-2 was obtained from Invitrogen (Carlsbad, CA). The stock solution was prepared by dissolving the non-sterile, lyophilized enzymes in Hank's buffered salt solution (HBSS) to the final concentration of 0.5% (w/v), then filter sterilized (0.2 μm) and stored at −20°C. For intravenous administration, the dose was 1 μl/g body weight. For the intratumoral injection of one tumor, only a fraction (the ratio of estimated tumor mass to body weight) of the intravenous dose was directly injected and the volume was limited to <50 μl to avoid the interference of introduction of the enzyme solution on the IFP. The other tumor in the same animal was used as the control and received an equal volume of deactivated collagenase (heat-denatured in 95°C water bath for 15 min). Thus, in this study two control groups were designed, one receiving intravenous collagenase-2 and the other one receiving intratumoral deactivated collagenase. Two collagenase dosages were used, 0.1% in the first set of tumors, and 0.5% in the second set of tumors with equal volume to investigate the concentration-dependent drug delivery change.

^{99m}Tc-Doxil^{TM} preparation and administration

Labeling of Doxil^{TM} with ^{99m}Tc was performed according to the methodology developed by Bao et al. [14]. In brief, 50 mg of sodium glucoheptonate (GH) (Sigma, St Louis, MO) and 3.5 μl of *N*, *N*-bis(2-mercaptoethyl)-*N*′,*N*′-diethylethylenediamine (BMEDA, ABX, Radeberg, Germany) were mixed in a vial, followed by the addition of 5.0 ml of saline for injection (nitrogen gas-flushed in advance). Following 20 min of magnetic stir mixing, 65 μl of freshly prepared degassed stannous chloride (Aldrich, Milwaukee, WI) solution (12 mg/ml) was added to the GH–BMEDA solution with the final pH adjustment of 7–8. Then, 1.0 ml of the resulting solution was transferred to a glass vial containing 1 ml of 2.96 GBq (80 mCi) ^{99m}Tc-pertechnetate (GE Healthcare, San Antonio, TX). The mixed solution was incubated at 25°C for 20 min with intermittent gentle shaking. Finally, 10 ml of Doxil^{TM} was added to the

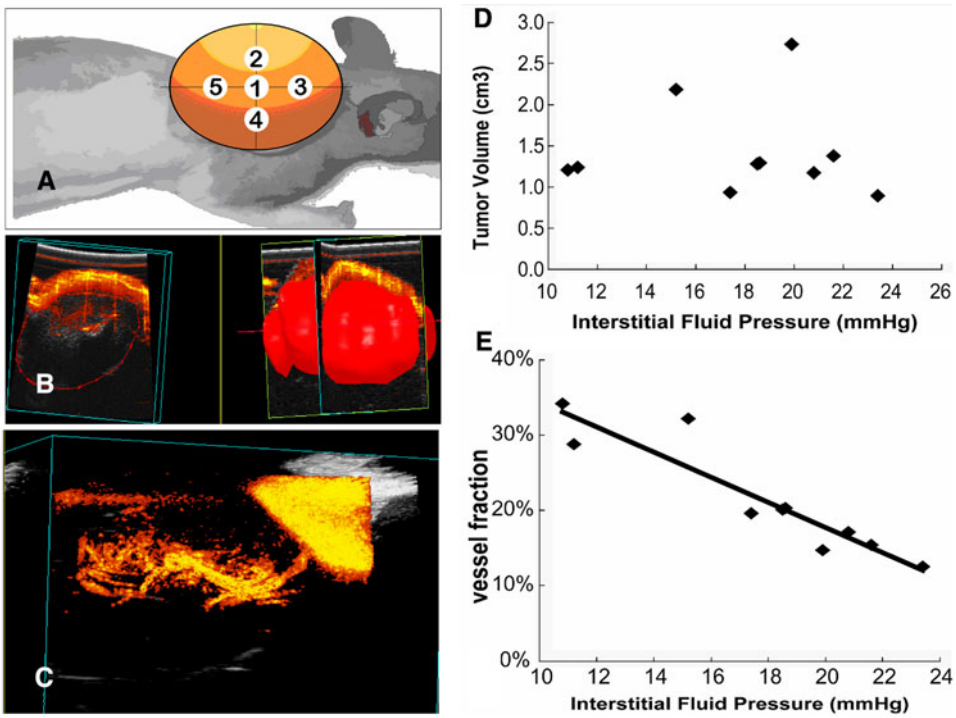

Fig. 1 Correlation between IFP and tumor volume, and vessel fraction. **a** IPF measurement point setting in HNSCC xenografts. Six measurement points were positioned at two perpendicular sections (Points 1 and 6 overlap). **b** Tumor was scanned with microultrasound in power doppler mode to simultaneously acquire the blood perfusion data and anatomical information. The tumor was reconstructed to calculate the tumor volume and vessel fraction. **c** Image of tumor blood vessels reconstructed based on the power doppler data. **d** and **e** Show plots of interstitial fluid pressure in xenografts against tumor volume and vessel fraction, respectively

^{99m}Tc-BMEDA solution and incubated at 37°C for 1 h. The ^{99m}Tc-Doxil™ was separated from unencapsulated ^{99m}Tc-BMEDA via disposable P-10 desalting column chromatography eluted with PBS buffer, pH 7.4. The labeling efficiency was 81.25 ± 4.31%. For each animal, ^{99m}Tc-Doxil™ was given intravenously 16-mg/kg lipid dose immediately following the intravenous injection of collagenase-2 or 30 min after direct intratumoral injection of collagenase-2.

Imaging evaluation with microultrasound and microSPECT

Microultrasound imaging system with a high frequency transducer (35 MHz, focal length 10 mm) was used to monitor tumor development by measuring the tumor size (length × width × depth) in B-mode images and tumor volume from reconstructed tumor 3D images. Power Doppler and pulse Doppler scanning modes were also used to evaluate blood perfusion and blood flow velocity. For power Doppler imaging, after 3D acquisition of tumor imaging, blood vessels were reconstructed using maximum intensity projection, and the vessel fraction (threshold at 1.5 cm/s) was derived automatically. To estimate the effects of collagenase on blood flow velocity, both peripheral and central vessels were sampled for pulsed Doppler imaging. For the intravenous collagenase use, the post-injection-pulsed Doppler imaging started at 30 min and for intratumoral use, at 120 min.

To estimate the Doxil™ accumulation and distribution, planar gamma camera images were acquired using a micro-SPECT/CT scanner (XSPECT, Gamma Medica, Northridge, CA) with dual CZT gamma cameras and parallel hole collimators 1 and 20 h after ^{99m}Tc-Doxil™ administration. Image data was transferred to workstation and processed using ASIPro image analysis software (Concorde Microsystems, Knoxville, TN). Once the imaging procedure was completed, the animals were exsanguinated under anesthesia and dissected. Tumors were excised, blot-dried with tissue paper, weighed, and counted in a Wallac 1480 Wizard 3" automatic well gamma counter (Perkin Elmer Life Sciences, Boston, MA) to measure the radioactivity accumulated in tumors. The percentage of the injected dose per gram tissue (%ID/g) for each tissue or organ was calculated using the following equation for the correction for differences in animal body weight and injected dose [15]:

$$\%\ \mathrm{ID/g} = \frac{\text{Tissue radioactivity (cpm)}/\text{Tissue wet weight (g)}}{\text{Radioactivity injected (cpm)}/\text{Body weight (g)}} \times 100\%$$

After completion of radioactivity measurement, selected tumors were used for autoradiography. Consecutive 2-mm-thick slices were cut from the tumor center with a microtome blade and then placed on phosphor plates designed for autoradiography, which were kept in a black sealed box stored in −20°C freezer. Twenty-four hour later, the phosphor plates were scanned, using a DenOptix QST phosphor scanning system (Lake Zurich, IL).

Second-harmonic imaging and immunohistochemical assay

To directly examine the collagen fibers in tumors, second-harmonic images of collagen were acquired using Nikon confocal microscope (Nikon, Melville, NY). Two-millimeter-thick slices were positioned on coverslips. During the microscopic observation, the samples were maintained fully hydrated with 0.9% saline. The excitation wavelength was 800 nm, and the second-harmonic signal was received at 480 nm. All images were acquired with series mode and saved as stacks, which facilitated the dimensional observation of collagen fibers. Hematoxylin & eosin (H&E) staining, Masson'strichrome staining, and immunohistochemical procedure were based on the slices used for autoradiography to achieve a good tissue match for evaluation of the correlation of collagen and drug distribution. In the Masson'strichrome staining slides, collagen component was dyed blue. For immunochemical staining, primary antibodies including rabbit-anti-rat collagen type I polyclonal antibody (Millipore, Danvers, MA), Endothelial cell antibody and LYVE1 antibody (Catalog# ab9774 and ab10278; AbCam, Cambridge, MA) were used. The working antibody concentrations followed manufacturers' recommendation. H&E and Masson's trichrome slides were examined with a light microscope, while the immunohistochemical slides were with a Zeiss confocal microscope (Carl Zeiss, Inc., Thornwood, NY) in the optical imaging facility. The images were analyzed with Image J (http://www.nih.gov).

Statistical analysis

All quantitative measurements are expressed as the standard deviation of the mean. Significance levels between groups were analyzed by either the student-*t* test or one way ANOVA using SPSS10.0 statistical software (SPSS Inc., Chicago, IL). The differences were considered significant when P value was <0.05.

Results

IFP was related to the vessel fraction instead of the tumor volume

IFP at different depths were recorded in ten tumors. The mean IFP values from all tumors were plotted against

tumor size and the vessel fraction calculated from B-mode ultrasound and the power Doppler scan, respectively (Fig. 1b, c). There was no defined relationship between IFP and the tumor size. The IFP in the smallest tumor with volume of 1.012 cm^3 was 24.6 ± 2.1 mmHg, while IFP in the largest tumor (2.875 cm^3) was 20.3 ± 2.1 mmHg. Only 2 tumors had relatively low IFP in the range of 11–12 mmHg (Fig. 1d). The average IFP in all tumors was 18 mmHg (±3.7). Clearly, there was a reverse correlation between IFP and vessel fraction ($r = -0.91$, $P = 0.009$). In tumors with higher IFP, the vessel fraction was lower, and vice versa (Fig. 1e). This finding could be partially attributed to the vessel distortion or closeness as a result of increasing interstitial pressure. The average vessel fraction was 21.4% (±7%).

Effects of collagenase on interstitial pressure

The response of tumor IFP to collagenase-2 administration is shown in Fig. 2. Intravenous administration of collagenase-2 induced continuous IFP decline within the first 35 min. From a pre-injection value of 30 mmHg, IFP decreased by 40% to 18 mmHg at 35 min. By 2 h, IFP recovered about 85% to 25 mmHg and 90% to 27 mmHg at 24 h. In contrast, the direct injection of the enzyme caused a spike in the IFP value from the pre-injection 25 mmHg to 40 mmHg at the end of the injection (first minute). Then the pressure went down and reached the initial value at 30 min. At 120 min, the pressure was recorded as 16 mmHg (35% reduction), the lowest value among all measured time points; and at 24 h, it had slightly recovered to 18 mmHg, still 30% lower than the initial value. Compared to the control intravenous route, the method of direct intratumoral injection could induce a similar reduction in IFP but had the advantage of maintaining the IFP at a lower level for a longer period.

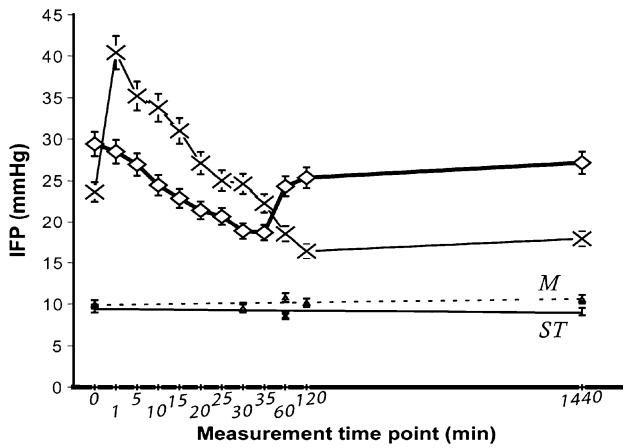

Fig. 2 Effects of collagenase-2 (0.1%) on the tumor interstitial pressure. Collagenase type 2 was administered intravenously or intratumorally, respectively. The interstitial pressures in muscle (*M*) and subcutaneous soft tissue (*ST*) were measured at selected time points as the reference

Another observation of the collagenase effect was on the blood flow within tumors. Theoretically, decreasing IFP will improve the blood perfusion and alleviate stasis, which could be reflected in the blood flow velocity. In this study, we sampled both peripheral and central vessels. Before intratumoral collagenase use, the blood flow velocity in the sampled central and peripheral vessels was 3.2 ± 0.4 and 5.1 ± 0.4 cm/s, while at 120 min, it was measured as 6.3 ± 0.4 and 7.0 ± 0.5 cm/s with 100 and 40% increase, respectively (Fig. 3).

Imaging study of ^{99m}Tc-Doxil^{TM} accumulation in tumors with SPECT

The main aim was to investigate by scintigraphic imaging the effect of collagenase use on the accumulation of the liposomal drug, ^{99m}Tc-Doxil^{TM} in tumors after intravenous administration, dynamic monitoring, and quantitative assessment of the drug distribution throughout the whole body, including in tumors. The SPECT images at 1 h did not show significant radioactivity difference at tumor sites between tumors with intratumoral injection of collagenase and the control tumors either with intratumoral deactivated collagenase or intravenous collagenase. In delayed SPECT images at 20 h, tumors with intratumoral collagenase showed high radioactivity accumulation compared to control tumors (Fig. 4a, b).

These findings were further confirmed by the calculation of the %ID/g and the autoradiography of dissected tumors. Figure 4c shows the calculated %ID/g from two experiments with different concentrations of collagenase. With both collagenase doses, %ID/g in tumors receiving intratumoral collagenase was significantly higher than the controls ($P = 0.02$ for 0.1%, $P = 0.007$ for 0.5%). The higher collagenase use (0.5%) increased the %ID/g to 107.32% compared to the 62.09% from the initial dose (0.1%) in tumors with intratumoral injection, while the higher dose did not induce significant changes in the control tumors. Additionally, no significant difference was observed between the controls. Also, no statistical radioactivity difference was observed between major organs including liver, spleen, kidney, muscles, and brain, between the testing group and control groups. The autoradiography of tumor sections provided a direct examination of the distribution of the ^{99m}Tc-Doxil^{TM}. Compared to the autoradiographic images from the controls (Fig. 5), the image from the tumor treated with intratumoral collagenase had multiple areas of high radioactivity accumulation.

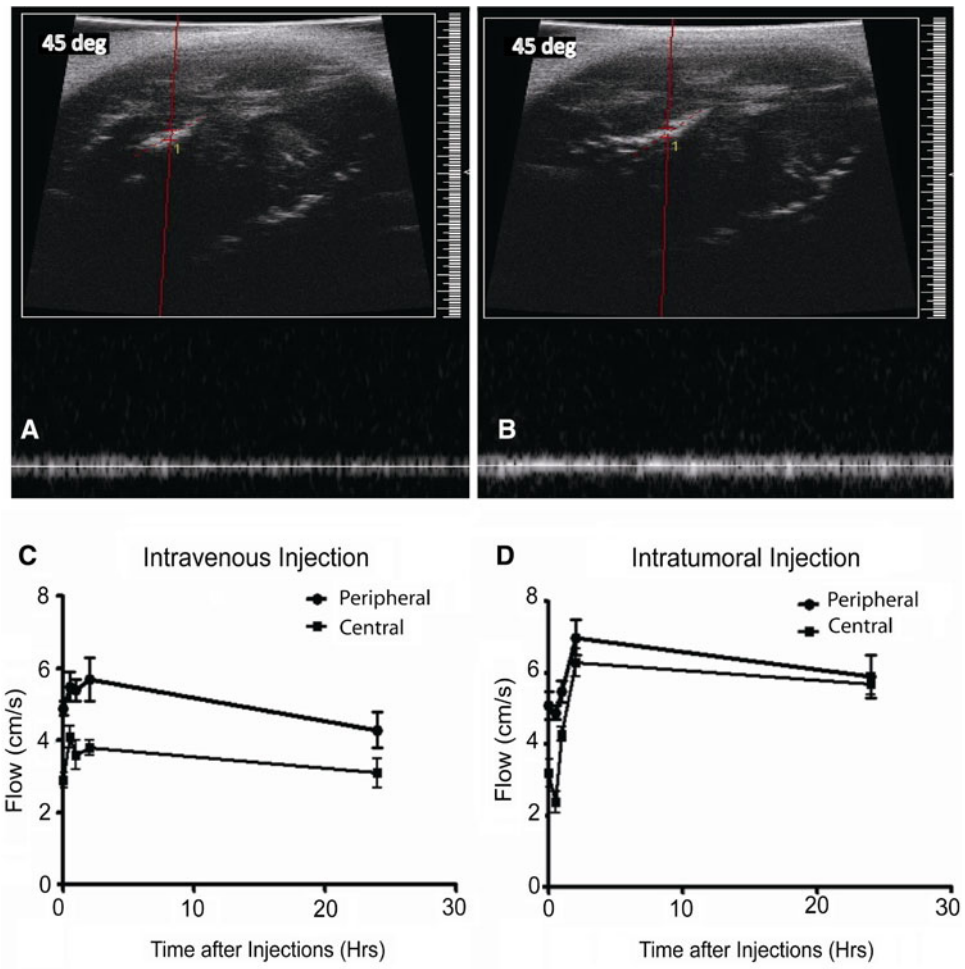

Fig. 3 The effect of intravenous and intratumoral collagenase-2 use on tumor blood flow in tumors. Pulse Doppler images of a central vessel were acquired **a** before and **b** 25 min after intratumoral use of collagenase-2. The *upper row*, the ultrasound image; the *lower row*, the blood flow strip. **c** and **d** Showed the change of blood flow velocity of central and peripheral vessels measured at various times after intratumoral and intravenous injection of collagenase-2

Microscopic examination of the effect of collagenase use on tumors and Doxil™ distribution

Second-harmonic imaging was able to disclose the formation of collagen in tissues. Collagen in the periphery of HNSCC tumor was highly dense and irregularly intertwined (Fig. 6a), while 24 h after administration of collagenase-2, the collagen meshwork got loose due to the degradation of collagen fibers, especially fine fibers with many coarse and thick fibers remaining (Fig. 6b). Once the autoradiography procedure was completed, tumor sections were processed for immunohistochemical staining with collagen 1 antibody and endothelial cell antibody. Combined with the autofluorescence feature of Doxil™, Doxil™'s microscopic distribution could be assessed directly. Compared to tumors from control groups, microscopic findings in tumors with intratumoral active collagenase injection had three representative features. (1) More open vessels can be visualized, which improved tumor blood perfusion (Fig. 6c). This observation was proved by a quantitative analysis by counting vessels with patent cavities filled with blood components. In tumors with intratumoral active collagenase, approximately twelve vessels (12 ± 4) were visualized per high-field view under microscope, compared to 7 and 6 in tumors with intravenous collagenase and intratumoral deactivated collagenases, repectively ($P = 0.2$). (2) The microscopic findings also showed segmented collagens with loss of fibrillar formation (Fig. 6d). and (3) There was a more extensive distribution of Doxil™ (Fig. 6e).

Discussion

In solid tumor treatment, an increase in drug delivery to the tumor and enhanced drug penetration within the tumor interstitium are fundamental requisites for effective eradication of tumor cells. Increased interstitial fluid pressure (IFP), a hallmark of solid tumors, hampers both drug delivery and drug penetration. As a response to this physiologic challenge, developing effective approaches to modulate the tumor IFP is justified.

In this study, we proposed to intratumorally inject purified collagenase-2 to reduce the collagen amount,

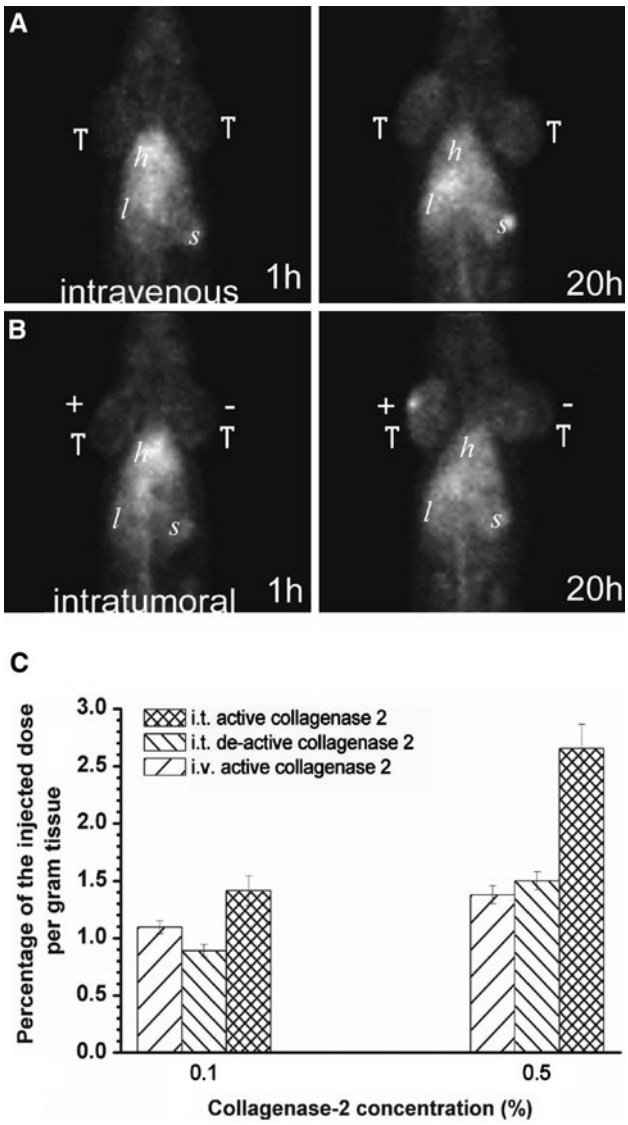

Fig. 4 Effects of collagenase-2 (0.1%) on the accumulation of ^{99m}Tc-Doxil^{TM} in tumors (*arrow*). Before intravenous ^{99m}Tc-Doxil^{TM}, collagenase-2 was administered intravenously (**a**) in four rats (including eight tumors) and intratumorally (**b**, active collagenase [+] to right tumors and deactivated [−] to left tumors in six rats), respectively. Planar gamma images were acquired at 1 and 20 h. *T* tumor; *h*: heart; *l*: liver; *s*: spleen. Collagenase concentration-dependent drug accumulation in tumors measured by relative tumor drug delivery was shown in (**c**). Two concentrations (0.1 and 0.5%) were used. Thereafter, ^{99m}Tc-Doxil were given intravenously and relative tumor drug delivery (i.e. % ID/g) was calculated after 20 h as described in "Materials and methods"

thereby decreasing the IFP and improving the drug extravasation and diffusion within tumors. Collagenases have three subtypes, collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13), according to the cleavage site on the alpha chain of fibrillar collagens [16]). Collagenase-1 uses type I, II, III, and IV collagens as substrates. The degradation of type IV collagen, known as the vascular basement membrane component, gives rise to the metastatic possibility through vascular and lymphatic systems. The same problem applies to collagenase-3. Due to this potential for metastatic-induction, attempts to use collagenase-1 or collagenase-3 in the clinical setting have drawn cautions and concerns. As described in the Introduction section, collagenase-2 secreted from neutrophils and endothelial cells is the collagenase that most efficiently initiates cleavage of type I collagens and has little role on the degradation of type IV collagens, which renders it as a better choice for tumor collagen degradation.

Several investigators have reported intravenous administration of collagenase to modulate tumor IFP. In a study using osteosarcoma xenografts (9), collagenase reduced both IFP (by 45%) and microvascular pressure (MVP, by 60%), but the kinetics of pressure modulation differed, as MVP had recovered by the time IFP reached its minimum level. Thus, collagenase increased the transcapillary pressure gradient. Also, collagenase treatment has been reported to increase the penetration and diffusion of monoclonal antibodies in tumors (9). A similar result was also observed in human ovarian tumors after administration of collagenase via the intraperitoneal route (10). Our intravenous data was consistent with these previous reports. With intravenous and intratumoral collagenase use, we measured IFP reduction by a maximum of 40 and 35%, respectively. However, in our experiment, the low tumor IFP in animals treated with intravenous collagenase remained for a shorter period (less than 2 h) than the tumor IFP in animals receiving intratumoral collagenase (>24 h). This result might be partially attributed to collagenase inactivation. Collagenases are quite sensitive to inactivation by metal chelating agents, such as cysteine or EDTA, and can also be inhibited by plasma α2-macroglobulin and α1-antitrypsin [17, 18]. The shorter period of low tumor IFP from intravenous collagenase could be further accountable to the insignificant effects on Doxil^{TM} accumulation compared to the deactivated enzyme, which together support our decision to apply the enzyme by an intratumoral route over an intravenous method. Furthermore, the other indirect evidence was the finding that a fivefold higher dose of intravenous collagenase did not produce better Doxil^{TM} delivery compared with intratumoral collagenase (from 62.09 to 107.32%).

Several techniques have been established for tumor IFP measurement, including wick-in-needle and micropipette [9, 19]. In this study, we used a Samba fiber-optic pressure recording (FOPR) system to record the IFP. Previous experience in small animals and pediatric patients validated its accuracy in pressure measurement with little drift and temperature sensitivity, especially the good stability for low-pressure measurements, which guaranteed the reliability and reproducibility of the Samba fiberoptic method

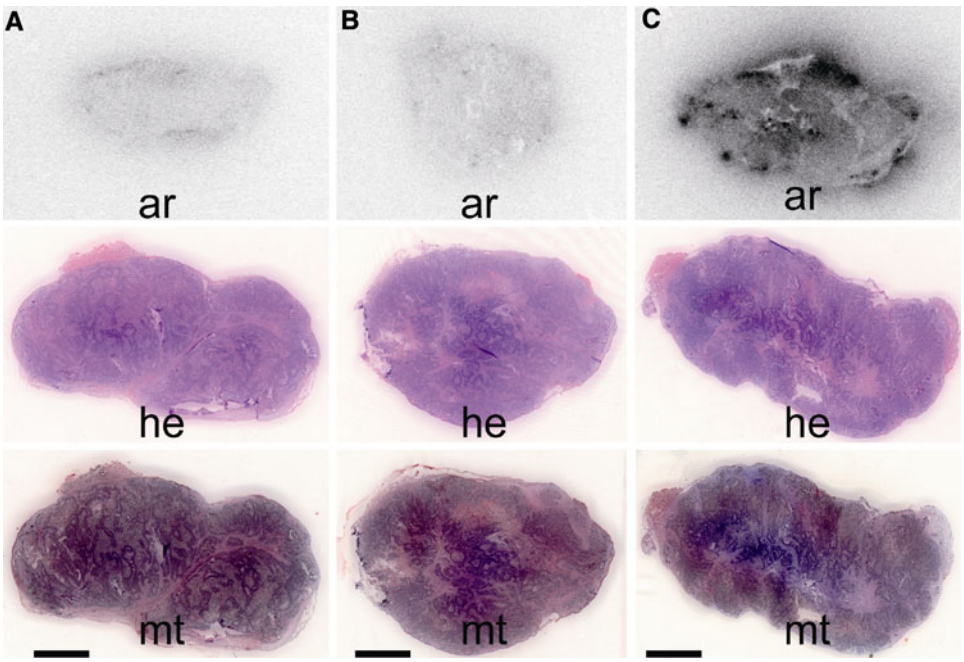

Fig. 5 The comparison of autoradiography (ar) and H&E staining (he), Masson's trichrome staining (mt). All tumor samples were collected from the tumor center with 2-mm thickness. Tumor slices were exposed to phosphor plates for autoradiography, then sent to pathology laboratory for H&E and trichrome staining and the immunohistochemical process. **a** From tumor receiving intravenous collagenase-2; **b**, from tumor with intratumoral deactivated collagenase-2 and **c** with intratumoral active collagenase-2 (0.1%). *Bar*, 3 mm. The experiment was repeated three times. Each time four rats (8 tumors) were used for the group of intravenous active collagenase, six rats (6 tumors) for the groups of intratumoral deactivated and active collagenase, respectively. Twenty hours following intravenous Tc^{99m}-Doxil administration, tumors were resected and sliced to 3-mm-thick tissue blocks for autoradiography. Then, the same tissue blocks were prepared for H&E, Masson's trichrome staining, and second-harmonic generation observation

in this study [20, 21]. The IFP in our HNSCC xenografts was around 20 mmHg (20 ± 2 mmHg), which was close to the reported measurement and not affected by the tumor age (in the observation period 5–20 days) or size [3, 9].

Vessel fraction from power Doppler ultrasound has been proven as a useful parameter to estimate the early-stage tumor response to tumor treatment [22]. The negative correlation between vessel fraction and IFP also indicated that this parameter could be employed to evaluate the IFP between different tumors or from the same tumor but at different time points.

In spite of the favorable IFP modulation results, intratumoral collagenase-2 use has its own limitations. The direct injection procedure requires the accessibility of tumors, which makes superficial HNSCC tumors better candidates. So, the indications for this treatment will be much narrower than those using an intravenous approach. The other worthy-to-be-mentioned limitation is the risk of metastasis through the needle tract, a concern also seen in tumor biopsy procedures. To minimize this risk, the operator can apply either a needle with as small gauge as possible or specially designed needles with the function of cauterization of the needle track that can stop minimal hemorrhage in the needle track and, more importantly, kill possible tumor cells attached on the needle to avoid the implantation to the adjacent normal tissues when withdrawing the needle [23].

With the increasing availability imaging modalities and the sophisticated imaging quality, image-guided therapy draws more enthusiasm in clinical community. Meanwhile, image-guided biopsy has been widely implemented in order to gain histological evidence for more specific therapy. As an invasive procedure, the biopsy procedure creates a direct route with a catheter to the lesion to harvest tumor specimens. Besides tissue sampling, the route could be used for intratumoral drug delivery without additional invasive approach. Without the modulation of the high interstitial fluid pressure in extracellular matrix, direct infusion of chemotherapeutical agents has limited effects due to inhibited diffusion. However, collagenase could degrade the integrity of the matrix and subsequently therapeutic agents given either intravenously or intratumorally would achieve better diffusion and higher accumulation, as proven in this study. Besides superficial head and neck tumors as we studied, deep-seated tumors, such as retroperitoneal masses, can also be managed with collagenase for better drug accumulation since imaging-guided invasive procedures bear few risks as a mature operation.

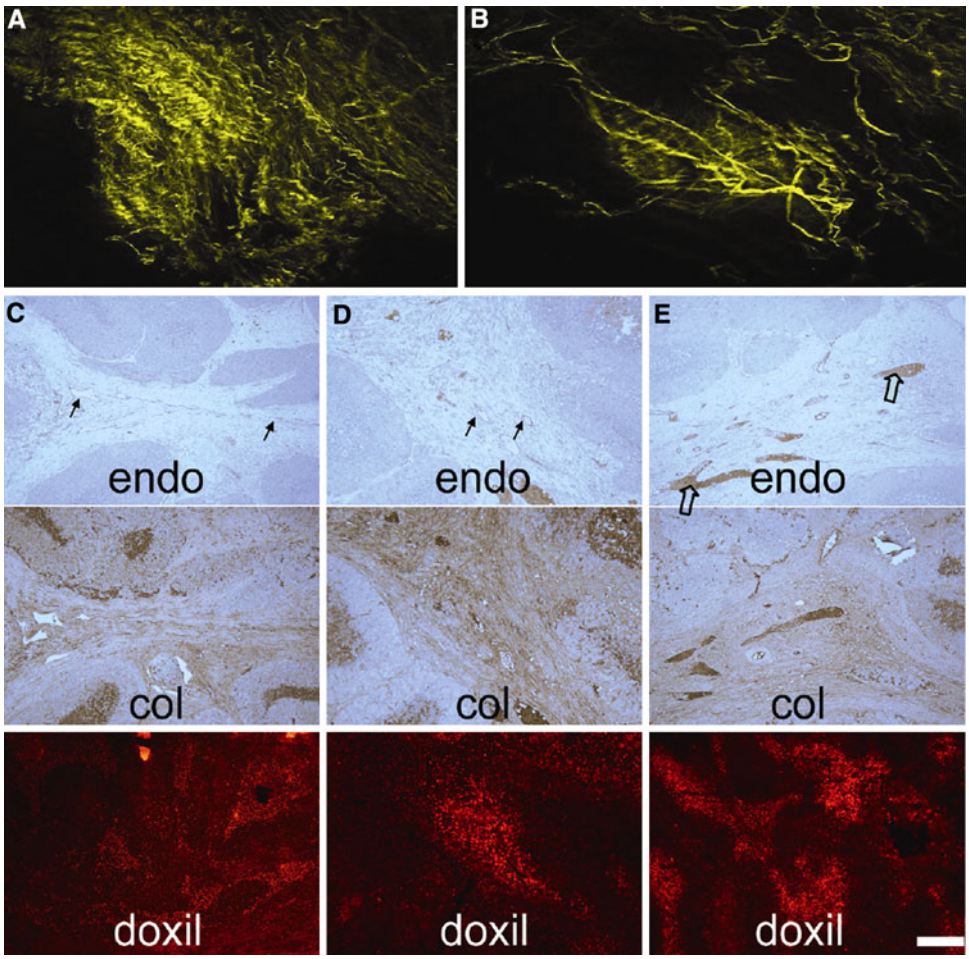

Fig. 6 Microscopic examination of the effect of collagenase-2 use on collagen with second-harmonic generation imaging and on vessels (endo, *brown*), collagens (col, *brown*), and Doxil distribution (Doxil, *red*) with immunohistochemical assay. **a** and **b** were the second-harmonic imaging of collagen in tumor tissues without and with intratumoral collagenase-2 application, respectively. **c** Tissue sample from tumor receiving intravenous collagenase-2; **d** tissue sample from tumor with intratumoral deactivated collagenase-2; **e** tissue sample from tumors with intratumoral active collagenase. Numerous closed or nearly closed vessels (*black arrows*) were shown in tumors without the treatment of active collagenase, while many open vessels (*open arrow*) were found in tumors after active collagenase-2 administration. Also, intratumoral injection of collagenase reduced collagen content and broke its fibrillar formation. Compared to the limited distribution of Doxil in **c** and **d**, Doxil in **e** spread into tumor cell nests. Image J (NIH.gov) was employed to analyze area of red particles, which was correlated to the distribution of doxorubicin. The result showed that at a preset color threshold, the red area in E (doxil) was 13.9%, while 7.3% and 6.6% in **c** (doxil) and **d** (doxil), respectively. 100X, *Bar*, 5 μm

Taken together, intratumoral administration of collagenase-2 had the capability of effectively reducing tumor IFP and maintaining the low IFP level for a long period to allow drugs to accumulate and diffuse within tumor interstitium within the limited tumor size range tested. Thus, with the tumor IFP modulation, a better therapeutic outcome can be expected and the drug availability to tumors will be increased so the actual drug loading dose may be accordingly adjusted to a small amount.

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Conflict of interest statement None.

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