Characterizing Extravascular Fluid Transport of Macromolecules in the Tumor Interstitium by Magnetic Resonance Imaging

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Abstract

Noninvasive imaging techniques to image and characterize delivery and transport of macromolecules through the extracellular matrix (ECM) and supporting stroma of a tumor are necessary to develop treatments that alter the porosity and integrity of the ECM for improved delivery of therapeutic agents and to understand factors which influence and control delivery, movement, and clearance of macromolecules. In this study, a noninvasive imaging technique was developed to characterize the delivery as well as interstitial transport of a macromolecular agent, albumin-GdDTPA, in the MCF-7 human breast cancer model in vivo, using magnetic resonance imaging. The transport parameters derived included vascular volume, permeability surface area product, macromolecular fluid exudate volume, and drainage and pooling rates. Immunohistochemical staining for the lymphatic endothelial marker LYVE-1 was done to determine the contribution of lymphatics to the macromolecular drainage. Distinct pooling and draining regions were detected in the tumors using magnetic resonance imaging. A few lymphatic vessels positively stained for LYVE-1 were also detected although these were primarily collapsed and tenuous suggesting that lymphatic drainage played a minimal role, and that the bulk of drainage was due to convective transport through the ECM in this tumor model. (Cancer Res 2005; 65(4): 1425-32)

Introduction

The interstitial or extracellular matrix of the tumor presents a barrier against the transport of macromolecules and against cancer cell dissemination. The ability to image and characterize the transport of macromolecular agents through the extracellular matrix or tumor interstitium can be employed to understand factors affecting this transport, their role in macromolecular drug delivery and in cancer cell dissemination. Because the ease of transport of macromolecules through the matrix can be used as an index of its integrity, in vivo imaging can be used to design strategies for altering the porosity and integrity of the interstitial matrix.

The past few decades have seen extensive development of noninvasive imaging modalities for characterizing the tumor vasculature in animal models (1, 2) and in patients (3). However, there have been relatively few noninvasive imaging methods for characterizing macromolecular movement through the extracellular matrix. In normal tissue, macromolecules and cell debris are cleared from the interstitium through lymphatic drainage. In tumors, lymphatics have either been shown to be poorly developed (4) or nonfunctional due to the existing mechanical stresses (5), with the result that the transport of macromolecules and cell debris through the extracellular matrix are thought to occur primarily via convective flow (6, 7). Several recent studies have transformed our understanding of the role of this interstitial-lymphatic continuum of tumors, from a passive to a more active one (8–10). These include the role of interstitial fluid flow in remodeling the extracellular matrix (11), as well as the meticulous molecular and functional characterization of the interstitial-lymphatic continuum (9, 12, 13).

Prior approaches for probing transport within the extravascular space, consisting of the interstitium and lymphatics (Fig. 1), have included (i) the use of fluorescence redistribution after photobleaching in situ (14), (ii) microlymphangiography (i.e., the injection of labeled macromolecules into the tumor interstitium and detection of its eventual uptake into the lymphatics), by multiphoton laser-scanning microscopy (4), (iii) micro-MR lymphangiography using dendrimer-based magnetic resonance imaging (MRI) contrast agents (15), and (iv) MRI using biotinylated BSA-GdDTPA (16).

In this study, we have shown, for the first time, the ability of MRI of the macromolecular contrast agent (MMCA) albumin-gadolinium diethyltriamine penta-acetic acid (GdDTPA) to quantify delivery, transport rates, and volumes of macromolecular fluid flow through the interstitial-lymphatic continuum in tumors. Immunofluorescent microscopy of a lymphatic endothelial cell–specific marker LYVE-1 (lymphatic vessel endothelial hyaluronan receptor; ref. 17) together with a vascular endothelial cell–specific marker GSL-1 was done to determine the presence and contribution of lymphatic vessels to macromolecular drainage within the tumor. The MRI technique described here bridges the gap between traditional intravital lymphangiographic approaches and multiphoton laser-scanning microscopy, by providing relatively high spatial resolution (100-250 μm) for visualization of interstitial transport and drainage in deep-seated, optically nontransparent tumor tissue in vivo.

Materials and Methods

Tumor Model and Inoculations. MCF-7 cells were inoculated in the upper left thoracic mammary fat pad of five female severe combined immune deficient mice. Tumor cells were inoculated in a volume of 0.05 mL. Hanks balanced solution (Sigma, St. Louis, MO) at a concentration of 106 cells/0.05 mL. All experimental animal protocols were approved by the Institutional Animal Care and Use Committee.

General Magnetic Resonance Imaging Paradigm. Mice were imaged 4 to 5 weeks postinoculation with tumor volumes in the range of 151.5 ±
111.7 mm³. MRI was done on a Bruker Avance 4.7-T instrument equipped with shielded gradients, using a custom-built RF volume coil placed around the body of the animal. Before placing each animal in the magnet, its tail vein was catheterized for administration of the MMCA albumin-GdDTPA. Because mice were imaged up to 140 minutes inside the magnet, an additional catheter containing the anesthetic solution was inserted s.c. for injecting additional anesthetic as required. One of the animals was imaged using biotinylated albumin-GdDTPA synthesized at the Weizmann Institute.

Multislice relaxation rate ($T_1^{-1}$) maps of the tumor were obtained using a saturation recovery method combined with fast-T1 SNAPSHOT FLASH imaging (flip angle = 10 degrees and echo time = 2 ms), as previously described (18). Images of six to eight slices (slice thickness of 1 mm) of the entire mouse cross-section including the tumor were acquired (128 × 128 matrix, 32-mm field of view, number of averages = 8) for each slice. These MRI acquisition variables translate into an in-plane spatial resolution of 250 × 250 μm². A multislice map of the completely relaxed magnetization ($M_0$ map) was also acquired once at the beginning of the MR experiment, using a recovery delay of seven seconds. Images were acquired in two "phases" corresponding to the biphasic kinetics of the MMCA (Fig. 2). The first or "early phase" comprised of images obtained before i.v. administration of 0.2 mL of 60 mg/mL albumin Gd-DTPA in saline (dose of 500 mg/kg) and repeated every 7 minutes, starting at 3 minutes postinjection, up to 31 minutes. Because drainage of macromolecules in and around tumors either by convection or by the lymphatics is a slow event (19–21), the second block of MR data was acquired up to 140 minutes postcontrast. This second block of acquisitions was classified as the "late phase" of the MMCA, as it consists of late drainage events within the extravascular space of the tumor. At the end of the study, the mice were sacrificed and blood $T_1$ values determined from blood samples taken from the tail vein (Fig. 2B).

Parameters describing the vascular and extravascular transport of the MMCA in MCF-7 tumors were calculated from the MMCA concentration-time curve [$\Delta R_{Tissue}(t)$] normalized to the MMCA concentration in the blood [$\Delta R_{Blood}(t)$], by assuming three distinct compartments within the tumor (Fig. 2). Analogous to a model described by Bjornea et al. (22), these three compartments are (i) the intravascular space, (ii) the perivascular region of the tumor interstitium that the MMCA initially extravasates into, and (iii) a more distant compartment consisting of regions of the interstitium within which slow macromolecular transport events such as convective and lymphatic drain occur. For both the "early" and "late phases" of the MR experiment, we modeled the MMCA uptake as a linear function of time (Fig. 2C and D). We then did a voxel-wise multiple linear regression analysis to determine the regression variables for each phase of the MRI protocol as described in the following sections.

Calculation of Vascular Variables (Analysis of Early Phase).

Assuming negligible reflux of the contrast agent and fast exchange for the duration of the MR experiment, MMCA uptake during the "early phase" (i.e., the first 31 minutes of the MR experiment) is given by:

$$\frac{C_{Tissue}(t)}{C_{Blood}(t)} = \frac{\Delta R_{Tissue}(t)}{\Delta R_{Blood}(t)} = V_V + \left(\frac{PS}{V_V}\right)t$$

(A)

Equation A is derived as follows:

Because we use an MMCA that is excluded from the intracellular space, $C_{intracellular} = 0$ and $C = C_VV + C_{peri}C_{peri} + C_{drain}C_{drain}$. During the early phase of the experiment, we assume unidirectional transport of MMCA from the intravascular to the perivascular space and negligible transport (if any) of the MMCA from the perivascular to the drainage compartment (Fig. 2A). For the permeability surface area limited case, the rate of contrast uptake is limited by the PS of the vessel wall and is given by (23):

$$\frac{dC}{dt} = PS C_V$$

(C)

Solving Eq. C, gives:

$$C(t) = PS C_V t + \alpha$$

(D)

where $\alpha$ is the constant of integration. Applying the initial condition $C(0) = C_VV$, gives $\alpha = C_VV$, which when substituted into Eq. D gives $C(t) = PS C_V t + C_VV$ or equivalently:

$$\frac{C(t)}{C_V} = PS t + V_V$$

(E)

This is the same as Eq. A:

$$\frac{\Delta R_{Tissue}(t)}{\Delta R_{Blood}(t)} = \frac{C_{Tissue}(t)}{C_{Blood}(t)} = V_V + \left(\frac{PS}{V_V}\right)t$$

where $[\Delta R_{Tissue}(t)/\Delta R_{Blood}(t)]$ is the ratio of the change in the relaxation rate of the tissue to that in the blood measured (by MRI) at some time $t$.

As shown by several investigators (18, 24), Eq. A indicates that the bulk of the $T_1$ relaxation effect is initially proportional to the volume of the intravascular space since the contrast agent is confined to this space (Fig. 1A). The ratio of the change in relaxation rate of the tissue to that in
the blood extrapolated to time $t = 0$ (i.e., the $y$-intercept) provides fractional vascular volume $V_V$ ($\mu$L/g) in that voxel (Fig. 2C). As the contrast agent extravasates into the perivascular space with time (Fig. 1B), the rate of change of the relaxation rate is proportional to the permeability surface area product; $PS$ ($\mu$L/g min) for that vessel (i.e., the slope of the concentration-time curve) is equivalent to the permeability surface area product, $PS$ (Fig. 2C). In other words, Eq. A is the equation of a straight line with slope $PS$ and $y$-intercept $V_V$ (Fig. 2C). Voxel-wise multiple linear regression analysis of this phase yields spatial maps of tumor $V_V$ and $PS$. Only those voxels for which the individual model variables $V_V$ and $PS$ passed the $t$ test for significance ($P \leq 0.05$) were considered for subsequent analysis.

**Calculation of Bulk Macromolecular Contrast Agent Transport Variables (Analysis of Late Phase).** Ninety to 140 minutes after the administration of the MMCA (i.e., for the "late phase" of the MR

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**Figure 2.** A, assuming negligible reflux of the contrast agent and fast exchange for the duration of the MR experiment, MMCA uptake was modeled as a linear function of time for the "early phase" (i.e., the first 31 minutes of the MR acquisition). For this phase, the slope ($slope_1$) of the concentration-time curve provides the permeability surface area product, $PS$ ($\mu$L/g min), and the $y$-intercept the vascular volume, $V_V$ ($\mu$g). During the early phase, we assume transport of MMCA from the vascular to the perivascular space is unidirectional, and that there is negligible transport (if any) of the MMCA from the perivascular to the drainage compartment. B, ninety to 140 minutes after the administration of the MMCA (i.e., for the "late phase" of the MR experiment), MMCA uptake was also modeled as a linear function of time. During this phase, it was assumed there was unidirectional transport of the MMCA from the perivascular to the slow drainage compartment with a flux rate, $FR$ ($\mu$L/g min) given by the slope ($slope_2$) of the late phase concentration-time curve. Schematic plot of the MMCA concentration-time curve over the duration of the entire MR protocol of (C) all the vascular variables computed from the early phase of the experiment and (D) all the extravascular variables computed from the late phase of the experiment, respectively. The discontinuity on the time axis is to emphasize the 60-minute interval during which there was no MR acquisition.
experiment), MMCA uptake is given by Eq. F:

$$\frac{C_{\text{Tissue}}(t)}{C_{\text{Blood}}(t)} = \frac{A_{\text{RatioTissue}}(t)}{A_{\text{RatioBlood}}(t)} = \beta_2 + (FR)t$$

(Eq. F)

Equation F is derived as follows:

Approximately 90 to 140 minutes after the administration of the MMCA (i.e., for the late phase of the MR experiment), it is assumed there was unidirectional transport of the MMCA from the perivascular space to the slower drainage space with a transport rate, $K_2$. For this phase, the rate of contrast uptake comprises of the continuing leakage (limited by the PS of the vessel) from the early phase as well as unidirectional transport from the perivascular to the drainage space with a flux rate, $K_2$ given by:

$$\frac{dC}{dt} = PSCV + K_2C_{\text{peri}}$$

(Solving Eq. G gives:

$$C(t) = PSCV_t + K_2C_{\text{peri}}t + \beta$$

where $\beta$ is the constant of integration. Applying the continuity condition that at the point of intersection ($t = t'$) of the early- and late-phase concentration-time curves, Eqs. A and H must be equal, leads to $PSCV_t' + C_0V_V - PSCV_t' + K_2C_{\text{peri}}t' + \beta$ (i.e., $\beta = C_0V_V - K_2C_{\text{peri}}t'$), which when substituted into Eq. H gives:

$$\frac{C(t)}{C_0} = \frac{C(t)}{C_0} = \left(PS + K_2 \frac{C_{\text{peri}}}{C_V} \right) t + \left( V_V - K_2 \frac{C_{\text{peri}}}{C_V} \right) t'$$

(Eq. K)

This can be written as:

$$\frac{C(t)}{C_0} = \left( PS + K_2 \frac{C_{\text{peri}}}{C_V} \right) t + \left( V_V - K_2 \frac{C_{\text{peri}}}{C_V} \right) t'$

If $(PS + K_2 \frac{C_{\text{peri}}}{C_V}) = FR$ and $(V_V - K_2 \frac{C_{\text{peri}}}{C_V}) = \beta_2$, Eq. K reduces to $\frac{C(t)}{C_0} = \frac{C_{\text{Ratio}}}{{C_{\text{Ratio}}}^0} \beta_2 + (FR)t$. This is the same as Eq. F.

Equation F is also the equation of a straight line with slope FR and $y$-intercept $\beta_2$ (Fig. 2D). Both these variables can be determined from the concentration-time data using multiple linear regression analysis. If the flux rate, $FR < 0$ we refer to FR as the efflux rate, because a negative slope is representative of drainage (i.e., elimination of the contrast agent from the voxel). If the flux rate, $FR > 0$, we refer to FR as the influx rate, because a positive slope is representative of pooling of the contrast agent within the voxel. Unlike transport from the vascular to perivascular space, which is an intravoxel event, the nonvascular transport from the perivascular to the drainage space occurs between voxels (i.e., it is an intervoxel phenomenon).

It is worth mentioning that (i) FR includes the continued extravasation of MMCA from the early phase as can be seen from Eq. K; (ii) during the early phase $C_{\text{peri}} = 0$, and Eq. K reduces to Eq. A; and (iii) the $y$-intercept ($\beta_2$) of the late-phase concentration-time curve (Fig. 2D) cannot be employed for determining the volume of the slow drainage compartment analogous to the determination of $V_V$ in Eq. A, as $K_2$ is unknown, and FR has a PS component (as can be seen from Eq. K). However, to further characterize this macromolecular transport in the interstitial compartment, we computed the apparent exudate volume, $V_E$ (mL/g), by multiplying the flux rate (FR) by the duration of the late phase.

Kinetics of Albumin-GdDTPA in Blood. Because characterization of extravascular transport required the mice to be imaged up to $\approx$140 minutes after i.v. administration of albumin Gd-DTPA, blood $T_1$ levels were determined over this period to ascertain that intravascular $T_1$ changes were not a source of changes in the MR signal in the tumor. In a separate

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**Figure 3.** A, MMCA concentration-time curve typical of a pooling voxel along with regression lines for the early and late phases of the experiment. The discontinuity on the time axis is to emphasize the 60-minute interval during which there was no MR acquisition. B, MMCA concentration-time curve typical of a draining voxel along with regression lines for the early and late phases of the experiment. C, three-dimensional cutaway of a postcontrast MR image of a MCF-7 tumor overlaid with a functional map showing the spatial distribution of the extravascular draining and pooling voxels. D, comparison of the %total number of tumor voxels identified as either being draining or pooling, for all the animals. Columns, mean; bars, ±SD.
study, described by us in ref. (25), blood $T_1$ values were quantified over the entire duration of this protocol using a customized small RF solenoid tail-coil system.

**Detection of Extravascular Macromolecular Drainage and Pooling.** Using the multiple regression approach described above, both early- and late-phase model parameters were simultaneously computed from the concentration-time data. Voxels within each slice were further classified as either being (i) “pooling voxels” (i.e., voxels in which the MMCA accumulated over time) or (ii) “draining voxels” (i.e., voxels from which the MMCA was eliminated over time). Based on our knowledge of MMCA drainage kinetics, if MMCA was being drained extravascularly from a voxel either by convection or via lymphatics, one should observe a decreasing or negative slope for the late phase concentration-time curve corresponding to elimination of MMCA (Fig. 1C). In terms of the four model variables ($V_V$, $P_S$, $\beta_2$, and $FR$), this is equivalent to the following condition: $slope_2 < slope_1$ or ($slope_2 - slope_1$) $< 0$ [i.e., ($FR - PS$) $< 0$]. In contrast, if the MMCA was pooling in the voxel, the slope of the late phase of concentration-time curve would be greater than that of the early phase [i.e., ($slope_2 - slope_1$) $> 0$, ($FR - PS$) $> 0$]. We tested for these unique conditions by subjecting the model parameters to the appropriate linear constraints during the data analysis stage. Results of a statistical test such as a general linear regression analysis by employing them in a general linear test for statistical significance. Consequently, only voxels for which the two conditions were statistically significant ($P \leq 0.05$) were classified as “draining” or “pooling” voxels. We then examined the values of the corresponding variables $V_V$, $P_S$, $FR$, and $EV_a$ for both these classes of voxels. The exudate flux rate $FR$, was referred to as the “influx” rate for pooling voxels and as the “efflux” rate for draining voxels, respectively.

Not only does the inspection of the spatial distribution of these two classes of voxels allow us to make inferences about the pooling/drainage kinetics of the MMCA but by inspecting $V_V$, $P_S$, $FR$, and $EV_a$ for the same, we can begin to parameterize the interstitial-lymphatic space. All MR images were analyzed using the Analysis of Functional Neuro-Images program AFNI (26). Histologic analyses of immunofluorescent antibody stained sections obtained from the imaged tumors were done to identify the presence of lymphatic vessels.

**Immunostaining.** Adjacent 5-μm-thick formalin-fixed, paraffin-embedded tumor sections obtained at 500-μm intervals were cut onto silanized glass slides, cleared of paraffin in Histoclear II (National Diagnostics, Atlanta, GA) and rehydrated by means of graded alcohol baths. After rinsing in double-distilled water, slides were transferred to a preheated (95-100°C) buffer of 0.1 mol/L Tris-HCl (pH 9.0), 2 mmol/L EDTA and heated in a microwave for 10 minutes at 95°C to 100°C, and then cooled for 30 to 45 minutes. Slides were then blocked in PBS 5% FCS for at least 15 minutes.

**Double Staining for Lymphatic Vessels and Blood Vessels.** Lymphatic vessels were detected using an antibody to the lymphatic endothelial hyaluronan receptor LYVE-1 (17). Briefly, slides were incubated overnight at 4°C with 4.7 μg/mL of rabbit anti-mouse LYVE-1 (provided by Dr. David G. Jackson) in saline, rinsed (×3) in PBS and incubated with goat anti-rabbit Alexa-Fluor 488 (Molecular Probes, Inc., Eugene, OR) secondary antibody for 90 minutes at room temperature. For detection of blood vessel endothelia, slides were incubated with the isoelectin, BS1-B4 from Bandeiraea simplicifolia conjugated to biotin (Sigma), for 60 minutes at room temperature after another PBS rinse. Slides were then washed again in PBS before being incubated with an avidin-TRITC (tetramethylrhodamine-isothiocyanate) conjugate (Sigma) for 60 minutes at room temperature. Slides were counterstained with Hematoxylin-2 (Richard Allan Scientific, Kalamazoo, MI), dehydrated in graded alcohol baths and mounted in an aqueous mounting medium, Faramount (Dako Co., Carpenderia, CA). The presence of green fluorescence in the tissue sections was used to identify lymphatic endothelial cells of lymphatic vessels, whereas red fluorescence was used to identify regions containing vascular endothelial cells and blood vessels. Finally, for animals that were imaged using biotinylated albumin-GdDTPA, following LYVE-1 staining slides were incubated with streptavidin Marina Blue (Molecular Probes) secondary antibody for 90 minutes at room temperature, followed by biotin blocking (blocking kit, Vector Laboratories, Inc., Burlingame, CA), followed by the previous protocol for staining blood vessel endothelia and counterstaining.

**Immunofluorescence Microscopy.** All slides were viewed (at 40×) using a Nikon ECLIPSE-TS100 microscope (Nikon Instruments Inc., Melville, NY) equipped with Plan-fluor lenses and filters for detecting GFP/FITC, RFP/TRITC and UV/4′,6-diamidino-2-phenylindole fluorescence. Regions of interest were digitized using a Nikon Coolpix 5000 digital camera (Nikon Co., Tokyo, Japan).

**Results**

Representative concentration-time curves analyzed using the multiple regression analyses are shown in Fig. 3. The average values of vascular volume and permeability surface area product of the MCF-7 tumors was $4.60 \pm 2.1 \mu L/g$ and $0.43 \pm 0.19 \mu L/min$ respectively. Figure 3A illustrates a voxel for which ($slope_2 - slope_1$) $> 0$ [i.e., $slope_2$ was significantly ($P \leq 0.05$) greater than $slope_1$]. Because such a concentration-time profile is representative of a voxel within which the MMCA is pooling, it was classified as a “pooling” voxel. Conversely, Fig. 3B illustrates a voxel for which ($slope_2 - slope_1$) $< 0$ [i.e., $slope_2$ was significantly ($P \leq 0.05$) smaller than $slope_1$]. Such a concentration-time profile is representative of a voxel from which MMCA is being eliminated during the late phase and was classified as a “draining” voxel. This analysis, carried out on a voxel-wise basis, enabled us to examine the three-dimensional spatial distribution of pooling and draining regions within the tumor as shown in Fig 3C. Overall, there was a greater percentage of pooling voxels (19.9 ± 16.8) identified compared with draining voxels (2.9 ± 1.5; Fig. 3D), and pooling voxels were often found in proximity to draining voxels (Fig. 3C). In addition, a paired t test indicated that the influx rate (1.31 ± 0.28 μL/g min) was significantly ($P = 0.001$) greater than the efflux rate (−0.28 ± 0.17 μL/g min) for all the imaged tumors (Fig. 4A). Although the PS of the draining regions (0.87 ± 0.54 μL/g min) tended to be higher than that of the pooling regions (0.25 ± 0.16 μL/g min; Fig. 4B), this difference was not significant. In contrast, the $V_V$ of the pooling regions (7.68 ± 1.55 μL/g) was significantly ($P = 0.001$) higher than that of the draining regions (3.68 ± 1.88 μL/g; Fig. 4C). Finally, there

**Figure 4.** Summary of the extravascular variables determined for all the animals in this study: A, comparison of efflux and influx rates for draining and pooling voxels, respectively. B, comparison of the mean permeability surface area product for draining and pooling voxels. C, comparison of the mean vascular volume for draining and pooling voxels. D, comparison of the bulk exudate volume for draining and pooling voxels, respectively. Columns, mean; bars, ±SD.
was also a significant difference ($P = 0.001$) in the apparent exudate volume of the pooling voxels (36.63 ± 7.70 µL/g) compared with the draining voxels ($-7.90 ± 4.72$ µL/g; Fig. 4D). All values presented are the mean ± SD. Figure 5 illustrates an example of interstitial drain occurring at the tumor-host tissue interface. Blood and lymphatic vessels were immunofluorescently labeled in the same tumor sections using a biotinylated primary antibody against lectin stained with avidin-TRITC, and LYVE-1 primary antibody stained with Alexa-Fluor 488, respectively. Detection of green fluorescence in these sections indicated the presence of lymphatic endothelial cells and lymphatics, whereas red fluorescence was observed in regions containing vascular endothelial cells and blood vessels. Each tumor section was also counter-stained with hematoxylin. Results from the immunohistochemistry studies obtained from a tumor are shown in Fig. 6. Lymphatic vessels exhibiting green fluorescence were observed in the tumor margins (Fig. 6A) with virtually no overlap with TRITC-stained blood vessels. Almost no intratumoral lymphatics were detected in the central regions of tumors. At high magnification ($100×$), the sparsely distributed lymphatic vessels at the tumor periphery were often seen to be intussuscepted by tumor cells (Fig. 6B-E). Figure 7 illustrates the intratumoral distribution of the biotinylated albumin-GdDTPA. Several MMCA-containing blood vessels are apparent in Fig. 7A, including areas wherein MMCA has extravasated. In contrast, no MMCA-containing lymphatic vessels were detected in any of the tumor sections.

**Discussion**

In this study, we have described a novel MRI paradigm to image and characterize delivery of MMCA through the vasculature and its transport within the tumor interstitium. We modeled the tumor to
The MMCA transport between compartments was modeled both, as a linear function of time and as consisting of an early phase and late phase. Our biphasic, multiple regression analysis approach was based on known dynamics of lymphatic-convective clearance of macromolecules within the tumor interstitium (16). Multiple linear regression analysis of each voxel was done to determine intra- and extravascular variables from the early and late phase of the MRI protocol, respectively. The advantage of employing a multiple linear regression approach is that it not only allows simultaneous computation of vascular and extravascular variables, but also permits easy identification of voxels based on any a priori chosen transport criteria. This is achieved by transforming the required criteria into a constraint for a general linear test to be tested during the linear regression.

Values of $V_v$ and $PS$ derived from the early phase of the experiment were consistent with previously published data for this tumor model (18). Data from the late phase of the experiment were used to identify draining and pooling voxels and to quantify bulk volumes and rates associated with these voxels. Draining voxels exhibited significantly decreased enhancement, whereas pooling voxels exhibited significantly elevated MR signal enhancement during the late phase of the protocol, compared with the corresponding early phase. Pooling and draining voxels were often contiguous, with predominant drain occurring at or near the tumor-host tissue interface. Pooling regions exhibited higher $EV_v$ (i.e., volume of exudate) than draining regions, as well as a higher (in)flux rates compared with the draining voxels. These observations are consistent with the presence of a significant fraction of hyperpermeable vessels. These results are also consistent with those obtained in a study by Dafni et al. (16) in which VEGF165 over expression in C6 glioma tumors induced blood vessel hyperpermeability with an increased outward flux of contrast agent towards the tumor periphery. The total MR detectable volume of interstitial fluid an hour after the onset of the late phase can be approximated by the sum of the influx and efflux rates multiplied by 60 minutes [i.e., $(1.31 + 0.28) \times 60 \approx 0.1 \text{ mL/h}$, which is comparable to the elimination rate for tumor interstitial fluid of $\approx 0.2 \text{ mL/h}$ reported by other investigators (20). In addition, the smaller exudate volume together with a lower (ef)lux rate of MMCA from draining voxels shows that the rate and amount of macromolecular clearance from the tumor interstitium is low. Factors such as elevated interstitial fluid pressure, blood vessel permeability, hydraulic conductivity, blood pressure, and lymphatic functionality affect the transport of macromolecules within the tumor interstitium (7); both convective and diffusive mechanisms (6) eventually drive MMCA drain within the tumor. Fluid exudate moves through the interstitial matrix along pathways of least resistance (28); elastin fibers within the extracellular matrix are thought to act as prelymphatic channels through the connective tissue, directing macromolecules into the lymphatics (Fig. 1; ref. 29). Our results suggest that clearance of macromolecules through the interstitial matrix was not efficient in this tumor model, consistent with the few lymphatic vessels detected in these tumors.

As shown by us in an earlier study (25), the small decrease in $R_1$ (or $1/T_1$) of the blood measured over the duration of these experiments, ruled out changes in blood $R_1$ as a potential source of the changes in the MR signal measured during the late phase of the experiment. Thus, the identification of both pooling and draining regions using the approach described here was most likely tracing a nonvascular or extravascular event, such as the movement of

![Image](image-url)
macromolecules into a lymphatic, or the movement of macromolecules via convective transport toward the tumor periphery. Furthermore, the time course of these observations is also consistent with previous reports of macromolecular transport characteristics through the tumor interstitium (16).

Although, the immunofluorescence studies revealed the presence of a small number of lymphatic vessels mostly localized to the tumor periphery, consistent with the draining regions of interest identified by the MRI analyses, there was no colocalization of biotin-labeled MMCA and the LYVE-1 stain. The involvement of these peripheral lymphatics in MMCA clearance for this tumor type was most likely minimal. In addition, the low fractional area of LYVE-1 stained lymphatics in comparison to that of the draining regions of interest identified using MRI implies that the bulk of the interstitial MMCA drainage for MCF-7 tumors was by convection and not via lymphatics.

The dynamic, noninvasive, in vivo functional assay described here, provides a powerful tool for probing the interstitial-lymphatic continuum and can be used for applications in which alterations in interstitial transport may occur. Such applications can include probing the remodeling of the extravascular space in tumors that often accompanies therapeutic interventions, and/or improving the delivery and transport of anticancer drugs.

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References

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