Published in final edited form as:

NMR Biomed. 2014 September ; 27(9): 1063–1069. doi:10.1002/nbm.3157.

In vivo detection of the effects of preconditioning on LNCaP tumors by a TNF- α nanoparticle construct using MRI

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Abstract

The outcome of systemic and local therapies (e.g., chemotherapy, radiotherapy, surgery, focal ablation) for prostate cancer can be significantly improved by using tumor-specific adjuvants prior to treatment (“preconditioning”). We propose to use dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) to monitor the in vivo response of a mouse model of prostate cancer treated with a vascular disruptive agent, TNF- α , delivered on a gold nanoparticle (NP-TNF). Six male nude mice bearing 4–5 weeks old LNCaP tumors were scanned at 9.4T. DCE-MRI was performed two days before and 4–5 hours after treatment with NP-TNF. An intraperitoneal bolus of Gadolinium-DTPA (Gd) was administered and contrast enhancement was measured for 90 minutes. Concentration time curves of Gd were calculated and the area under the Gd curve (AUGC) was determined pre and post treatment. NP-TNF treatment caused an increase in contrast uptake in tumors. Interestingly, the early concentration (10 minutes post Gd bolus i.p.) was similar in both untreated and treated conditions; however, 90 minutes after injection, [Gd] was 3.4 times higher after treatment compared to before. AUGC doubled from 11 ± 6 [Gd] $\times$ min before treatment to 22 ± 9 [Gd] $\times$ min after treatment. An increase in signal enhancement was also observed in the muscle but to a lesser degree. We also evaluated the kinetics of intravenous gadolinium administration in mice bearing a jugular vein catheter to mimic the delivery method used in clinical trials. The overall treatment effects were independent of the delivery pathway of the contrast agent. In conclusion, we show that DCE-MRI is suitable to detect changes associated with a vascular disruptive agent in a mouse model of prostate cancer. The ability to characterize the effects of nanoparticle therapy in vivo with non-destructive methods is important as such compounds, in combination with treatment strategies, progress towards clinical trials.

Keywords

DCE; nanoparticles; cancer; prostate; preconditioning; preclinical

Introduction

The vasculature of solid tumors presents features that are not present in normal vessels. Specifically, tumors exhibit higher vessel density due to extensive angiogenesis, increased vascular permeability and defective vascular architecture. These alterations result in increased extravasation, which, along with an impaired lymphatic clearance, cause the accumulation of macromolecules and lipids specifically in the tumor tissue. This effect, called the “enhanced permeability and retention” (EPR) effect [1,2], makes the tumor vasculature an ideal candidate for targeted molecular therapies [2–4].

Using a nanoparticle that exploits and potentiates the EPR effect prior to systemic or local therapy has been shown to improve outcome in animal models, by increasing tumor sensitivity to subsequent treatments [3,4]. In particular, it was shown recently that the intravenous administration of a molecule combining the vascular disruptive agent, TNF- α , delivered on a gold nanoparticle (namely, CYT-6091, or NP-TNF) improved cryosurgical outcomes in a mouse model of prostate cancer by “preconditioning” the tumor prior to the procedure [4,5]. Tumor necrosis factor α (TNF- α) is highly toxic when injected systemically, precluding its straightforward use in humans; however, combining with gold nanoparticles decreases its toxicity, while increasing the specificity of action to the tumor [6,7]. Preconditioning by the TNF- α / gold construct induces a series of physiological events such as a decrease in local perfusion and an increase in nanoparticle accumulation in the tumor (potentiated by the EPR effect), in vascular permeability, in interstitial space volume and in the local inflammatory response [6]. Effects of CYT-6091 in vivo has been monitored in the tumor using a dorsal skinfold chamber (DSFC), an approach allowing direct visualization of the tumor tissue and the surrounding vessels by inserting a “window” directly over the tissue of interest [8]. However, this technique is not suitable for measuring the effect of NP-TNF in patients, and it does not allow the monitoring of the effect of NP-TNF in surrounding tissues and structures.

As more efficient therapies involving adjuvant preconditioning are developed, clinically relevant methods to monitor the effects of these agents on the tumor are needed to evaluate dose effect, optimize timing between adjuvant administration and treatment and possibly provide patient-specific timing and dose determination [9,10]. Dynamic contrast enhanced MRI (DCE-MRI) is a candidate method to monitor the activity of vascular disruptive molecules and nano-drugs on the vasculature. Specifically, DCE-MRI permits the determination of parameters reflecting blood flow and vessel permeability [11] routinely performed in clinical settings for cancer detection, and has also been shown to be sensitive in monitoring treatment effects in humans [12].

We recently showed the potential of DCE-MRI to monitor preconditioning in a mouse model of prostate cancer [7,13]. In this work, we aimed at non-invasively and quantitatively characterizing the effect of preconditioning by the TNF- α / gold nanoparticle construct CYT-6091 in mice implanted with human LNCaP prostate tumors using DCE-MRI. We also evaluated two different protocols of contrast agent administration, namely intraperitoneal and intravenous, for the characterization of NP-TNF effect in this rodent model.

Materials and Methods

Tumor implantation

LNCaP Pro 5 human prostate cancer cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum, antibiotics, and 10^{-9} mol/L dihydrotestosterone as previously described [5]. Cells were then suspended in 100 μ l of Matrigel matrix (BD Biosciences) and 1 million cells were injected on each hind limb of 5 athymic nude mice (20–25 g, Jackson Laboratory). Tumors were allowed to grow for 4 to 5 weeks (size $\sim$ 7–10 mm diameter) before imaging. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

CYT preparation

The TNF- α and gold nanoparticle construct, CYT-6091 (NP-TNF), was provided by Dr G. Paciotti (CytImmune Sciences, Rockville, MD). The content of one vial (100 mg) was diluted using 2 mL of sterile water and freshly prepared daily.

Animal preparation

Figure 1 shows the two experimental protocols used in this study. The first protocol, using an intraperitoneal injection of contrast agent, was used due to its relative easy technical realization, and allowed the same animals to be imaged both pre and post treatment. The second protocol was used to achieve a reliable intravenous administration of contrast agent, closer to human studies and clinical conditions; however, because of the technical difficulties of this protocol in mice, animals were separated in two groups including those receiving NP-TNF and controls. In the first protocol (figure 1A) animals were scanned twice, on two separate days. On the first day, no NP-TNF was administered allowing baseline measurements of the tumor to be obtained. Animals were allowed to recover for 24 hours after the first imaging session, and were scanned again on the third day 4 hours after an intravenous injection of 100 μ l of NP-TNF via the tail vein. Injection of NP-TNF was performed under ketamine/xylazine anesthesia (150/50 mg/kg i.p., respectively). Animals recovered fully after about one hour, and the MR session was started 2 to 3 hours after NP-TNF injection. An intraperitoneal catheter was positioned for gadolinium-DTPA (Gd) injection (0.6 mmol/kg using a 0.1 M Gd solution). In the second protocol (figure 1B), a jugular vein catheter was surgically implanted in 10 mice anesthetized with ketamine/xylazine. Animals received post-operative care during the 2-day recovery, and on the day of the experiment were separated in two groups, the control group and the treatment group ($n = 5$ in each group). For those in the treatment group, NP-TNF was administered using the jugular vein catheter under isoflurane anesthesia for which recovery was immediate, and the scanning session was performed 2 to 3 hours after NP-TNF injection. Gadolinium (0.6 mmol/kg using a 0.1 M Gd solution) was administered via the jugular vein catheter. Note that the gadolinium injection was performed $\sim$ 2 hours after the start of the animal preparation for the MR session, such that the effect of NP-TNF as measured by DCE-MRI was 4–5 hours post NP-TNF. For all imaging experiments, the animals were anesthetized using isoflurane (1.5–2%) in O_2/N_2O (50/50). Body temperature was maintained using warm air. Respiration and temperature were monitored throughout the experiment.

Imaging protocol

All imaging experiments were performed using a 9.4 T/31 cm bore magnet (Magnex) equipped with a Varian/Agilent Technologies console. A commercial volume coil (Millipede, Agilent, Palo Alto, CA) was used for RF transmission and reception. After acquisition of scout images, data were acquired for the computation of B_1 maps using a 3D-gradient echo sequence at 2 different repetition times for transmit power optimization [14]. Prior to contrast injection, a multi-slice gradient echo sequence was acquired at 7 different flip angles (from 2 to 40°) to calculate T_1 maps (FOV = 35 mm, matrix size = 128×128 , 1 mm thickness, TE = 1.43 ms, TR = 65 ms, 4 averages, acquisition time 39.8 s). The same acquisition sequence was used to acquire DCE-MRI data to follow contrast kinetics, both pre and post nanoparticle administration, with the following parameter changes: 8 averages, acquisition time per volume 79.6 s, FA = 30°, total acquisition time = 1h 29min for ip, and 1h 46 min for iv.

Data processing

B_1 maps were processed using in-house routines developed in Matlab (The MathWorks Inc., Natick, MA, USA). Dynamic contrast enhancement curves were obtained after the conversion of the images into DICOM format using a Matlab routine, and were then processed using the Osirix software (<http://www.osirix9-viewer.com/>) [15]. The mean and standard error of the percent signal enhancement was determined over the time course of the DCE-MRI studies for all tumor regions from all mice in order to compare the differences in contrast dynamics with and without NP-TNF administration.

For individual mice, parametric maps showing the integrated area under the Gd concentration-time (AUGC) curves were generated to visualize the spatial accumulation of Gd. The time course data was first converted to concentration using the pre-contrast T_1 maps [16,17] followed by integration over the entire time course on a pixel-wise basis in IDL (Exelis Visual Information Solutions, Boulder, CO, USA). Regions of interest determined on the corresponding grey-scale images were used to assess contrast uptake differences in both tumor and muscle. Pre- and Post-TNF concentrations and AUGC were compared using a two-tailed, unpaired Student's t-test, with $p < 0.05$ considered as threshold for significance.

Results

Figure 2A shows an axial view of a typical mouse bearing LNCaP tumors after intraperitoneal Gd injection. Figures 2B and 2C show the percent change in signal intensity following Gd injection averaged over all tumors and muscle respectively acquired before and after NP-TNF experiments. The Gd bolus was given 5 minutes after the start of the experiment. In the tumor, contrast uptake is enhanced after NP-TNF administration (Post-NP-TNF) compared to the uptake measured before NP-TNF administration (Pre-NP-TNF). Also note that the shape of the curves in the initial period after injection (first 10 minutes) suggests a slower uptake after NP-TNF treatment. In the muscle, contrast also increased after NP-TNF treatment, however it plateaus around 60 minutes, whereas it still increases in tumors within the observation window (>90 minutes). Figure 3 shows typical Gd concentration maps acquired in a mouse 5, 20 and 80 minutes after bolus administration.

Figure 4 shows AUGC maps before and after NP-TNF, where the accumulation of Gd is clearly higher in the Post-NP-TNF tumor. Figure 5 shows the concentrations of Gd derived from the intensity curves at 4 time points (10, 20, 50 and 90' after beginning of the experiment, i.e. 5, 15, 45 and 85' after contrast injection), showing that the concentrations of gadolinium is comparable between both conditions early after the bolus is given, and increases more than five-fold 85 minutes post bolus. A summary of the results is presented in Table 1. Figure 6 shows the results obtained after intravenous administration of Gadolinium. In Figure 6A, an axial view of a mouse after intravenous injection of contrast agent is shown. Figure 6B shows the change in signal intensity in the control (blue symbols) and NP-TNF (in red) groups, in the muscle and in the tumors. Note that although the curves profiles are different from the ip route for contrast agent injection, the main effect (increase in signal, Gd accumulation, following NP-TNF treatment) is comparable between the two protocols employed, with a faster washout of contrast following iv injection of Gd as expected. Figure 6C and D shows the AUGC and [Gd] maps, respectively, indicating a higher accumulation of Gd within the tumor tissue.

Discussion

In this work, we non-invasively measured the effect of a TNF- α construct on tumors using DCE-MRI in a mouse model of prostate cancer. The changes in perfusion and contrast retention found in the tumor confirm its potential impact on treatment when used prior to or concomitant with other definitive therapies.

DCE-MRI in the LNCaP model

Using an intraperitoneal bolus of contrast agent, we observed a complex dynamic pattern of enhancement. In the first ten minutes after contrast agent injection, the uptake is lower in treated vs. untreated tumors. Past this initial period, the contrast accumulates significantly more after NP-TNF treatment. The NP-TNF construct used in this study has been shown to induce a drop in perfusion between 2 to 6 hours after injection [6] during which time we acquired the DCE MRI data (between 4 and 5.5 hours post-NP-TNF treatment). The slower initial uptake is likely due to this drop in perfusion. The later increased enhancement likely reflects the EPR effect [7], further enhanced by increased permeability due to TNF- α activity [18]. Notably, the intraperitoneal route for injection of contrast agent yielded a slower contrast loading than the intravenous route. In this condition, the DCE MRI acquisition protocol is therefore more sensitive to physiological events evidenced by late enhancement as opposed to first-pass, such as necrotic processes including cell membrane rupture observed in hepatic tumors for example [19]. It is likely that a complex combination of these processes (increase in vessel permeability, slower extravasation from EPR and cell membrane rupture) contributes to the large increase in contrast uptake after NP-TNF treatment [7].

We focused on parameters that are model-independent for the analysis of the DCE-MRI data. We chose to do so as the estimation of other parameters describing permeability and exchange characteristics of the tissue (such as K^{trans} , v_e or k_{ep}) can be inaccurate if the appropriate model and arterial input function (AIF) are not used [20]. The acquisition of a

good quality AIF is challenging in mice but can be done by acquiring data simultaneously in the left ventricle [21,22], however the coil employed in this study did not have sufficient coverage. As an alternative approach, the AIF can be acquired in a separate group of animals, and the resulting population-averaged AIF can then be used for a model-based analysis. We performed studies in both the IP and IV mice in order to derive population AIFs for use in pharmacokinetic modeling (data not shown). While the AIF could be used to derive reasonable pharmacokinetic parameters for tissues with “native” vasculature (i.e. the muscle), the neovasculature of the heterotopic tumors had both a delayed enhancement and slower enhancement kinetics [23,24]. As such, with an AIF acquired from the native vasculature or blood pool, it was not appropriate or even possible to perform pharmacokinetic modeling in the implanted tumor, at least with standard two-compartment models. Finally, an alternative method is to use the muscle as a reference region [25]; however, we show that the muscle physiology is also affected by NP-TNF, thereby preventing a similar model to be used in both conditions. To avoid the complications of using the model based fitting in this study, we focused on the gadolinium concentrations and resulting AUGC calculations which are both quantitative and highly sensitive for characterizing the effect of NP-TNF in tumors, and represent a simple, unbiased and robust measure.

Because MRI (as opposed to optical imaging methods) allows observing multiple organs at the same time, we could also observe effects of NP-TNF on the muscle. This indicates that NP-TNF still has a systemic effect despite its demonstrated preferential loading in tumor tissue [26]. Newer versions of the drug are under development, aiming at further improving the local delivery aspect of this nanodrug. On a side note, this observation suggests the use of MRI as shown here as an ideal preclinical tool in animal models, in which systemic effects of drugs can be imaged and evaluated.

Moving forward to clinical use: limitations, challenges and advantages

In this study, we used an animal model of prostate cancer in which the tumors are large and not localized in situ. Admittedly, other models, such as orthotopic implantation models, would better reflect human tumor progression with respect to metastasis [27] and to the fact that the tumor microenvironment depends on the site of implantation [28]. However, the use of the presented subcutaneous model of LnCAP was motivated by the fact that our group has extensively characterized the distribution and other characteristics in previous histology and functional studies [4,7,26,29]. It remains to be demonstrated whether the response of the tumors to NP-TNF demonstrated here is observed for in situ tumors in particular in the case of small disease. However, if the extensive contrast accumulation observed in tumors is confirmed in human studies, NP-TNF pretreatment could also have an additional role in helping localize small tumors in patients.

Whether the contrast agent was injected intraperitoneally or intravenously, the dynamic curves obtained from the LNCaP tumors exhibit vastly different kinetics than observed in human cancer, i.e. the data were acquired over more than one hour, while it takes approximately 5 minutes for the contrast to wash in and out in human cancers [30]. Repeated experiments cannot therefore be performed on the same day, as observed in a

previous study [31]. We first chose the intraperitoneal route due to the technical challenge represented by repeated intravenous injection in the caudal vein of the mouse. Because of our study design, three iv injections would be needed over the course of two days: a first one for gadolinium before NP-TNF treatment; a second one for NP-TNF administrations; and a third one for the second DCE-MRI acquisition, the second and third being on the same day. In practice, the mouse caudal veins are easily damaged by repeated injections, preventing subsequent experiments. In contrast, intraperitoneal injections are easily repeated and allowed us to perform a longitudinal study in the same animals. With an implanted jugular vein catheter we could perform two consecutive IV injections; however, this approach is challenging and requires a great deal of technical expertise. We found that the jugular vein catheters easily clogged after a single scanning session, such that two separate groups were required. The effect of NP-TNF was qualitatively comparable between the two protocols (i.e. IP versus IV). This comparison demonstrated that the relative impact between pre and post NP-TNF administration on the DCE-MRI kinetics were similar. The IP studies provided the ability to observe the same mouse under both conditions and IV studies provided the kinetics to be observed with a contrast delivery route more similar to that used clinically. Together, these results demonstrate the potential of DCE-MRI to be a potentially valuable tool in evaluating the impact of NP-TNF and potentially other nanoparticles in future clinical trials.

Moving forward to clinical translation of nanoparticles, an alternative to monitor changes in blood flow using MRI is Arterial Spin Labeling (ASL). Despite its relatively low sensitivity, this method presents several advantages: it is not based on an exogenous contrast agent, and as such the technical challenges of intravenous injection are avoided, the experiment can be repeated several times without the concern for repeated contrast administrations, and would allow the measurement of the effects of NP-TNF on the perfusion in real time. In this particular study, ASL is challenging in animal model of LNCaP tumors due to the very low basal perfusion of these tumors, rendering the detection of a drop in perfusion (as opposed to an increase) difficult and likely inaccurate. However, ASL would be a possible technique to consider for human studies. The feasibility of ASL perfusion imaging in the prostate for clinical studies has been recently demonstrated at 3T [32], and studies at increasing field strengths (such as 7T) would even further improve the resolution and accuracy of such measurements in the prostate, due to the longer T_1 relaxation times yielding higher signal and greater labeling efficiency.

Conclusion

In conclusion, we demonstrated that DCE MRI is a useful and non-destructive tool for monitoring and quantifying the effects of NP-TNF on a tumor which we term: preconditioning. While previous studies have shown decreased perfusion and toxicity of the nanoparticle compared to native TNF- α , imaging with DCE-MRI has highlighted the presence of increased EPR effect and the presence of transient systemic effects. In general, this study supports the use of DCE-MRI as a non-destructive means to monitor changes in perfusion, permeability and retention as a result of nanoparticle delivery. As such, this may be a valuable tool to develop and characterize the development of similar agents in the

future and provide the necessary information to evaluate and optimize such agents in translational clinical trials.

Acknowledgments

This work was supported by NIH P41 EB015894 and AHC Translational Research Grant Program 2009 09-08. The authors want to thank Emily Colonna for expert technical support with animal preparation and monitoring. We also gratefully acknowledge CytImmune Sciences, Inc. for their generous gift of CYT-6091 for the studies and Dr. Joel Slaton for his intellectual support of this work.

Abbreviations

NP-TNF	gold nanoparticles/TNF- α conjugate
Gd	gadolinium
DCE	Dynamic contrast enhanced
EPR	enhanced permeability and retention

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Figure 1. Experimental protocols

In (A), the protocol used for an intraperitoneal injection of Gadolinium (Gd) is shown. In protocol (B), gadolinium was injected through a jugular vein catheter implanted in mice 2 days prior to imaging. Please note that in both cases NP-TNF was administered intravenously, using the caudal vein in A and the jugular vein catheter in B. Gd: Gadolinium.

Figure 2. Axial view of a typical mouse bearing tumors after Gd injection (A) and signal enhancement curves in tumors (B) and muscles (C)
 In (A), the green solid line delineates a tumor, and the yellow dashed line delineates a typical muscular region used for the analysis. In (B) and (C), the blue and red symbols represent the data acquired before and after NP-TNF treatment respectively. Data are presented as mean $\pm$ SEM ($n = 10$ tumors and 10 muscle regions). The bolus was given 5 minutes after the start of the acquisition. SI, difference in signal intensity.

Figure 3. Gadolinium concentration maps acquired before (Pre-) and after (Post-) NP-TNF injection

The images show the concentration maps of the same mouse before and after treatment with NP-TNF (axial view). The maps shown were acquired 5 min, 20 min and 80 min after injection of the contrast agent. The arrows point to the tumors implanted on each hindlimb.

Figure 4. AUGC maps acquired before (Pre-) and after (Post-) NP-TNF injection
The arrow shows a typical tumor implanted on the hindlimb. (A), typical AUGC map before NP-TNF treatment; (B), AUGC map acquired in the same animal after NP-TNF treatment.

Figure 5. Time course of the average concentration of gadolinium in tumors before (PRE) and after (POST) injection of NP-TNF

Data are presented as mean $\pm$ SEM (n = 10). The blue symbols represent the data acquired before NP-TNF treatment; the red symbols show the data acquired after NP-TNF treatment.

Figure 6. Summary of the results obtained using an intravenous route for gadolinium injection
 In (A), the green solid line delineates a tumor, and the yellow dashed line delineates a typical muscular region used for the analysis. In (B), the blue and red symbols represent the data acquired in the control and the NP-TNF groups respectively. The two upper curves are the mean signal change $\pm$ SEM in tumors, and the two bottom curves are the mean signal change $\pm$ SEM in adjacent muscles ($n = 10$ tumors and 10 muscle regions). The bolus was given 5 minutes after the start of the acquisition. In (C), a close up of the AUGC maps is shown. In (D), the concentration maps of gadolinium at 6, 20 and 90 minutes are shown.

Table 1

Average T_1 , gadolinium concentration and AUGC in mice before and after NP-TNF injection.

	Tumors (n = 10)		Muscle (n = 10)	
	PRE	POST	PRE	POST
T_1 (ms) (before bolus)	1577 $\pm$ 232	1650 $\pm$ 276	1313 $\pm$ 163	1435 $\pm$ 314
[Gd] (mM)				
20'	0.08 $\pm$ 0.04	0.08 $\pm$ 0.07	0.07 $\pm$ 0.02	0.09 $\pm$ 0.04
90'	0.15 $\pm$ 0.09	0.51 $\pm$ 0.15 ^c	0.02 $\pm$ 0.01	0.12 $\pm$ 0.05 ^c
AUGC				
([Gd] $\times$ min)	11 $\pm$ 6	22 $\pm$ 9 ^b	4 $\pm$ 1	10 $\pm$ 3 ^a

Average data obtained by manual delineation of regions of interest in tumors (2 tumors per mouse in 5 mice) and adjacent muscles. Data are shown as average $\pm$ SD. T_1 maps are acquired prior to the injection of the bolus of gadolinium. Gadolinium concentrations are shown at 20 minutes and 90 minutes after the beginning of the DCE-MRI acquisition, and the bolus was started ~3 hours after NP-TNF injection.

^a p=0.012,

^b p=0.006,

^c p=0.0005, Pre- vs. Post.