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Molecular Ultrasound Imaging of Tissue Inflammation Using an Animal Model of Acute Kidney Injury

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Abstract

Purpose—The objective of this study was to evaluate the use of molecular ultrasound (US) imaging for monitoring the early inflammatory effects following acute kidney injury.

Procedures—A population of rats underwent 30 min of renal ischemia (acute kidney injury, $N=6$) or sham injury ($N=4$) using established surgical methods. Animals were divided and molecular US imaging was performed during the bolus injection of a targeted microbubble (MB) contrast agent to either P-selectin or vascular cell adhesion molecule 1 (VCAM-1). Imaging was performed before surgery and 4 and 24 h thereafter. After manual segmentation of renal tissue space, the molecular US signal was calculated as the difference between time-intensity curve data before MB injection and after reaching steady-state US image enhancement. All animals were terminated after the 24 h imaging time point and kidneys excised for immunohistochemical (IHC) analysis.

Results—Renal inflammation was analyzed using molecular US imaging. While results using the P-selectin and VCAM-1 targeted MBs were comparable, it appears that the former was more sensitive to biomarker expression. All molecular US imaging measures had a positive correlation with IHC findings.

Conclusions—Acute kidney injury is a serious disease in need of improved noninvasive methods to help diagnose the extent of injury and monitor the tissue throughout disease progression. Molecular US imaging appears well suited to address this challenge and more research is warranted.

Keywords

Acute kidney injury; Contrast agent; Ischemia-reperfusion injury; Molecular imaging; Targeted microbubbles; Tissue inflammation; Ultrasound

Introduction

Acute kidney injury is a serious disease with grave consequences. Importantly, one of the major mechanisms involved in the pathogenesis of this disease is a transient drop in the blood supply to the kidney. It continues to be a problem that markedly affects outcome in critically ill patients, increasing length of hospitalization and health care costs and may also progress to chronic kidney disease. Despite recent advances in treatment options, development of acute kidney injury continues to be associated with high mortality rates that can range from 40 to 90 % [1, 2]. Moreover, acute kidney injury is characterized by a rapid decline in glomerular filtration rate over a period of hours to days and accompanied by the retention of waste products. This impairment in kidney function can be caused by a number of different factors and occurs in a significant fraction of patients in the intensive care unit. In addition to the clinical relevance of studies that examine acute kidney injury, experimental renal ischemia and reperfusion injury is also an important model used to assess the conditions that occur in patients receiving a kidney transplant. Depending upon the donor, transplanted kidneys are not perfused with blood for a variable amount of time prior to transplantation. Because acute kidney injury has serious effects in patients, and all transplanted kidneys experience renal ischemia and reperfusion injury to some extent [3], the clinical relevance and translational importance of this type of research to human health is extremely high.

Several different imaging modalities have been used to gather information on kidney anatomy (to rule out obstruction), differentiate acute from chronic kidney injury, and to obtain information on renal blood flow and glomerular filtration rates [4]. Contrast-enhanced computerized tomography (CT) and magnetic resonance imaging (MRI) are extremely limited because of the inherent patient risk associated with contrast-induced toxicity [5, 6]. An ultrasound (US) contrast agent (known as a microbubble, MB) is superior for imaging the renal vasculature because they are non-nephrotoxic and do not diffuse out of the vascular space. Under appropriate conditions, contrast-enhanced US can be used as a noninvasive imaging tool for collecting quantitative measurements of regional renal perfusion and microvascular function [7, 8]. With respect to the current and potential clinical applications, contrast-enhanced US is a useful technique in the evaluation of acute pyelonephritis, renal tumors, cystic lesions, vascular insults, and renal transplantation [9]. Contrast-enhanced US imaging may also be of value for monitoring renal tumor response to antiangiogenic treatment [10, 11]. While contrast-enhanced US was recently shown feasible for the early detection and monitoring of acute kidney injury [12], a more direct method for assessing the microvascular inflammatory response might prove useful for not only quantifying the degree of tissue injury but also for determining the impact of potential treatments.

Molecular US imaging has the potential to detect molecular changes before phenotypic changes become apparent and holds promise for the highly sensitive detection of disease biomarkers [13]. Similar to most other forms of molecular imaging, molecular US imaging relies on the detection of a reporter capable of providing signal enhancement reflecting some molecular process of interest [14–18]. Of particular importance, identification of inflammation-related receptors overexpressed in injured renal tissue can be exploited as targets for next-generation ligand-labeled MB contrast agents [19–21]. One general conjugation strategy has been to attach disease-specific ligands to the contrast agent shell surface via a high-affinity molecular bridge [22]. After injection, these ligand-labeled contrast agents systemically circulate, bind at the target tissue [23], and are readily imaged by contrast agent-specific US methods. In the present preclinical study, we evaluated the use of MBs targeted to known biomarkers of tissue inflammation [*e.g.*, P-selectin or vascular cell adhesion molecule 1 (VCAM-1)] for molecular US imaging in acute kidney injury. We hypothesized that an abundant retention of targeted MBs in inflamed renal tissue after intravenous administration would provide a means to image early inflammatory responses. We further hypothesize that there is a positive correlation between the molecular US signal and target biomarker expression. These hypotheses were tested in an established animal model by performing molecular US imaging at early time points after ischemia-reperfusion injury or sham surgery.

Materials and Methods

Animal Preparation

The Institutional Animal Care and Use Committee (IACUC) at our institution approved all experiments before initiation. Twelve male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) underwent complete right nephrectomy via a right loin incision using established surgical procedures and under aseptic conditions [24]. Thereafter, eight animals had a micro-serrefine vascular clamp placed to restrict all flow to the renal pedicle of the remaining kidney for a length of 30 min. The remaining rats underwent a sham treatment in which the remaining kidney was removed from the body cavity for the same duration of time but no vascular restriction was applied. In both cases, the kidney was kept moist with sterile gauze soaked in saline. Afterwards, the kidney was placed back into the body cavity and the wound was sutured closed. Following surgery, the rats were placed on a warming pad for 2 h to allow recovery from the surgical procedures.

Molecular Ultrasound Imaging

At baseline, animals that were scheduled to receive either acute kidney injury or sham surgery were divided into two equal groups. After hair removal, the animals were positioned on their right side, US coupling gel was applied over the kidney, and the US transducer was fixed in the transverse plane. Prior to image acquisition, each animal was slowly injected in the tail vein with either a 0.1 mL bolus of P-selectin (Visistar P-selectin; Targeson, San Diego, CA) or VCAM-1 (Visistar VCAM-1; Targeson) targeted MBs. Note the size (diameter) and stock concentration for each product was approximately the same, 2.4 μm and 2.65×10^6 MBs per mL, respectively. Immediately before injection and for 5 min thereafter, pulse-inversion imaging of targeted MBs was performed using a SONIX RP US

system (Ultrasonix Medical Corp, Richmond, BC) equipped with a L12-4 linear array transducer at a mechanical index (MI) of 0.1. The acoustic focus was set proximal to the left kidney while transmit and receive frequencies were 5 and 10 MHz, respectively. An intermittent frame rate of 0.1 Hz was achieved via an external trigger and used in part to minimize any targeted MB destruction due to US exposure [25]. In addition to a conventional grayscale US image for anatomical mapping, molecular US images were acquired in all animals at baseline and again at the 4 and 24 h time points thereafter. US scanner settings were fixed for all imaging sessions.

Image processing of all scan-converted (8-bit) US datasets was performed using custom Matlab programs (Mathworks Inc, Natick, MA). After all frame data is loaded for a particular animal, both grayscale and contrast-enhanced US images are displayed side-by-side for visual review. The kidney space is then isolated using manual segmentation (polygonal region-of-interest, ROI) from either image types. The resultant binary mask is applied to the time sequence of contrast-enhanced US images before derivation of a mean time-intensity curve from data in each segmented ROI. After a systemic bolus injection, targeted MBs will freely circulate and a small population will selectively bind to the intravascular inflammatory biomarker and progressively accumulate whereas the remaining unbound contrast agents will be rather quickly cleared from the blood pool given the fact that their half-life is on the order of minutes (Fig. 1). US images captured after clearance of unbound MBs can be normalized by the first frame in the sequence (before MB dosing) to remove background noise and yield a simple measurement or spatial map of the molecular US signal.

Immunohistochemistry

Following the 24 h time point, animals were humanely euthanized. Kidneys were removed, cut transversely approximately along the US imaging plane, and placed in 10 % neutral buffered formalin for 48 h before transferring to a 90 % ethanol solution for short-term preservation. Tissues were embedded in paraffin, and 5 μ m sections were stained with hematoxylin and eosin (H&E). Morphological assessment of injury was determined by examining for necrosis, casts, and areas of red blood cell extravasation into the interstitium. Analysis of P-selectin (CD62P) and VCAM-1 (CD106) expression was performed in addition to staining for presence of vascular endothelial cells (CD31). Established immunohistochemistry (IHC) methods were used to process all tissue samples. Slides were reviewed by light microscopy (Leica DMI6000 B inverted microscope; Leica Microsystems Inc, Buffalo Grove, IL) and captured using a high-resolution digital camera (Leica DFC310 FX; Leica Microsystems Inc). Targeted biomarker information was quantified from the digitized stained tissue sections and recorded as percent tissue area [26].

Statistical Analysis

Data was summarized as mean $\pm$ SE when applicable. A linear regression analysis was used to study relationships between the molecular US signal and immunohistochemistry findings. A repeated measures analysis of variance (ANOVA) test analyzed longitudinal intra-animal measurements whereas a two-sample *t*-test was used to assess differences between animal

groups. Data was analyzed using JMP Pro 10 software (SAS Institute Inc., Cary, NC) and a p -value less than 0.05 was considered statistically significant.

Results

Molecular US imaging was performed in animals subjected to 30 min of renal ischemia (*i.e.*, acute kidney injury) or control sham surgery. Derived from a 5 min series of contrast-enhanced US images of the segmented kidney space, Fig. 1 illustrates a representative time-intensity curve that reflects targeted MB contrast agent arrival, receptor binding, and progressive dissolution as freely circulating (unbound) MBs are cleared from the circulatory system. Note that after approximately 3 min, the time-intensity curve plateaus. Thereafter, the static difference in US image intensity from baseline measures ($t=0$) is attributed to bound MBs that have accumulated inside the renal vascularity and represents a generalized mean measure of the molecular US signal. A series of spatial maps describing the molecular US signal are detailed in Fig. 2. For animals injected with either P-selectin or VCAM-1 targeted MBs, there appears to be no difference in the baseline US images. However, within hours of acute kidney injury, the molecular US images exhibit considerable enhancement due to increased renal tissue inflammation, which is an observation not evident from review of the control animal images. Also note that targeted MB binding is more pronounced in the renal capsule (peripheral) compared to the medullary region. The molecular US signal in this example was reduced by the 24 h time point and presumably due to the re-establishment of renal perfusion and decrease in post-trauma inflammation. These molecular US signal measurement trends are further highlighted in Fig. 3, which summarizes all US findings for this study. At baseline, there were no differences in the molecular US signals collected from control and injured renal tissue for either the P-selectin ($p=0.74$) or VCAM-1 ($p=0.59$) targeted US imaging studies. For animals imaged with P-selectin or VCAM-1 targeted MBs, the molecular US signal increased by nearly 600 % ($p=0.04$) and 300 % ($p=0.03$) and within 4 h for the acute kidney injury group animals, which subsided to increases of about 110 % ($p=0.08$) and 90 % ($p=0.10$) by 24 h, respectively. No such changes in the molecular US signal were observed in the control animals regardless of the inflammatory target ($p>0.38$).

Immediately following the 24 h imaging time point, animals were humanely euthanized and the kidneys were prepared for IHC. The cross-sectional image plane was carefully noted during all renal US examinations so that all biomarker expression levels could be as closely matched as possible. Review of renal tissue sections revealed that after ischemic injury, P-selectin expression was present in the glomerular tufts and considerably upregulated the intrarenal vasculature (Fig. 4). Note that P-selectin staining was also detected in the glomeruli of control tissue sections so any biomarker levels found in the glomerular tufts of injured kidney could be basal expression. In comparison, VCAM-1 expression was increased predominately in the peritubular capillaries and endothelial cells in the larger renal blood vessels. These distinguishing patterns were noted as being uniform throughout each tissue section. Also detailed in Fig. 4, there was nearly a 30 % ($p=0.10$) and 40 % ($p=0.12$) increase in renal tissue P-selectin and VCAM-1 expression levels for animals that experienced acute kidney injury only 24 h prior. These biomarker expression levels had a strong positive correlation to the respective P-selectin ($R^2=0.67$, $p=0.05$) or VCAM-1 ($R^2=0.80$, $p=0.02$) targeted US imaging findings. While VCAM-1 expression levels are

more pronounced, further analysis of data plotted in Fig. 4 would suggest that molecular US imaging of the P-selectin biomarker is the more sensitive strategy for quantification of renal tissue inflammation following acute injury. While the data is not shown, there was also positive correlation between P-selectin and VCAM-1 expression levels that trended towards statistical significance ($R^2=0.25$, $p=0.10$).

Discussion

Acute kidney injury is characterized by a rapid decline in kidney function within a few hours to days. Different imaging modalities can potentially provide valuable information with respect to the anatomy of the kidney, possibility of obstruction, and renal hemodynamics. However, depending on the condition of the patient and the severity of the kidney injury, not all imaging modalities are suitable due to nephrotoxicity associated with contrast agent usage. In a majority of cases, renal US is the imaging study of choice due in part to its ease of use, safety profile, and widespread availability [4]. Given the relatively recent development of MB contrast agents, which have no nephrotoxic effect, the clinical utility of real-time contrast-enhanced US for monitoring renal hemodynamics is only now starting to be explored [27–30]. By providing a more detailed assessment of renal perfusion and the microcirculation, contrast-enhanced US has the potential to serve as a very useful tool in the diagnosis and management of patients with acute kidney injury [31].

In this study, we explored the combination of targeted MBs and contrast-enhanced US for molecular US imaging of renal inflammation following acute kidney injury. Using either a MB contrast agent targeted to P-selectin or VCAM-1, molecular US imaging in an animal model proved useful for detecting renal inflammation and within 4 h of exposure to ischemic conditions. Importantly, there was a positive correlation between the molecular US signals and biomarker expression recorded at the 24 h time point. Our data also suggests the molecular US imaging of intravascular P-selectin levels is a more sensitive strategy for assessing acute kidney injury compared to imaging upregulation of VCAM-1. Notwithstanding, histology findings indicated that P-selectin upregulation was primarily localized in the intrarenal vasculature after acute kidney injury whereas VCAM-1 expression was increased in the peritubular capillaries and endothelial cells in the larger intrarenal blood vessels. Given these rather unique patterns, future *in vivo* studies might explore the utility of a dual-targeted MB to both P-selectin and VCAM-1 since this strategy was shown to improve binding almost 2-fold during a series of *in vitro* flow chamber experiments [32]. The considerable gains in molecular US image enhancement from the *in vivo* use of multi-targeted MBs has been well documented [16, 18, 33, 34] and may further improve the already promising findings from our current study. Future studies should also include molecular US imaging with corresponding histologic assessment at earlier time points to determine if US can differentiate severity of kidney injury. This study should include known clinical measures of kidney function, such as renal blood flow and glomerular filtration rates, for comparative purposes.

The preclinical use of molecular US imaging to assess inflammation and renal tissue injury dates back more than a decade. In a seminal paper by Lindner et al., it was shown that MBs targeted to P-selectin produces a strong signal enhancement on renal US imaging of

inflamed tissue following ischemia-reperfusion injury [19]. It was also shown that the molecular US signal from P-selectin targeted MBs is more pronounced in the renal cortex compared to the medulla region following only 30 min of ischemia [21], which matches observations made from our study. While we evaluated individual MBs groups targeted to either P-selectin or VCAM-1, both exhibited a strong correlation to target biomarker expression.

Limitations of our study can be found in the small sample size used and the fact that the molecular US imaging strategies were not evaluated in the same animals (*i.e.*, P-selectin and VCAM-1 targeted US image data). The latter was due to technical difficulties in performing repeated MB injections in the same rat tail vein. Future studies utilizing vascular access ports might help alleviate this issue [35, 36]. While renal contrast-enhanced US imaging is primarily a modality that operates in two-dimensional space, previous work has shown that small changes in transducer position can produce substantial standard deviations in measurement repeatability [37]. It is conceivable that these same issues can manifest when using planar molecular US imaging. To that end, future work should explore molecular US imaging of the entire kidney (three-dimensional space). Lastly, it is important to note that serum creatinine levels were not recorded during this US imaging study. This biomarker is one of the primary tests used clinically to help diagnose acute kidney injury, which by definition is a rise in creatinine over a 24 to 48 h period [38]. Given known limitations in the diagnostic performance of serum creatinine changes [39], molecular US imaging of biomarkers for renal inflammation may prove more effective for the early diagnosis of acute kidney injury, but more studies are needed to validate these claims.

Conclusions

Acute kidney injury is a serious disease in need of improved methods to help diagnose the extent of injury and monitor the tissue throughout disease progression. Data and preliminary work described herein detailed noninvasive molecular US imaging of two different disease-related targets using an animal model of acute kidney injury. Results suggest that molecular US imaging of P-selectin expression appeared more sensitive than VCAM-1 expression. Histology findings indicated that P-selection upregulation was primarily localized in the intrarenal vasculature after acute kidney injury whereas VCAM-1 expression was localized to the peritubular capillaries and endothelial cells in the larger intrarenal blood vessels. Overall, there was a positive correlation between all molecular US measurements and inflammatory biomarker expression.

Acknowledgments

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Fig. 1.

Representative time-intensity curve that reflects targeted microbubble (MB) contrast agent arrival (wash-in), receptor binding, and progressive dissolution as freely circulating (unbound) MBs are cleared from the circulatory system (wash-out). After approximately 3 min, the time-intensity curve plateaus. Thereafter, the static difference in ultrasound (US) image intensity from baseline measures (ΔI) is attributed to bound MBs that have accumulated inside the renal vascularity and represents a generalized quantitative measure of the molecular US signal. A progressive sequence of contrast-enhanced US images are displayed and reflect targeted MB circulation and retention in the renal vascular space. Note that the frame color for each contrast-enhanced US image corresponds to the colored data point in the time-intensity curve (*i.e.*, average intensity of the segmented image).

Fig. 2.

Spatial maps of the molecular US signal color-coded and overlaid on grayscale US images. Within hours of acute kidney injury (ischemia), the molecular US images exhibit considerable enhancement due to increased renal tissue inflammation. Note that targeted MB binding at 4 h is more pronounced in the renal capsule region. After 24 h, the molecular US signal was reduced due to the injured kidneys recovering from short-term ischemic conditions.

Fig. 3. Summary of longitudinal changes in molecular US imaging measurements using either a P-selectin (a) or VCAM-1 (b) targeted MB contrast agent. Bar graphs detail differences between ischemic and control kidney data. (* $p < 0.05$).

Fig. 4. Representative histology images of renal tissue after acute kidney injury indicating that (a) P-selectin expression was localized in the glomerulus tufts (G) and intrarenal vasculature (V) whereas (b) VCAM-1 upregulation was localized to the peritubular capillaries (arrows) and blood vessel endothelium. Summary of histologic measurements at 24 h from the (c) P-selectin and (d) VCAM-1 targeted molecular US imaging study animals. (e) The scatterplots

detailing paired inflammatory biomarker expression and molecular US imaging measurements reveals positive linear relationships (*dashed lines; $R^2>0.67$, $p<0.05$*).

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