Ultrasound Assessment of Inflammation and Renal Tissue Injury With Microbubbles Targeted to P-Selectin

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Background—Routine methods capable of assessing tissue inflammation noninvasively are currently not available. We hypothesized that tissue retention of microbubbles targeted to the endothelial cell adhesion molecule P-selectin would provide a means to assess inflammation with ultrasound imaging.

Methods and Results—Phospholipid microbubbles targeted to P-selectin (MBp) were created by conjugating monoclonal antibodies against murine P-selectin to the lipid shell. The microvascular behaviors of MBp and control microbubbles without antibody (MB) or with isotype control antibody (MBiso) were assessed by intravital microscopy of cremasteric venules of control and tumor necrosis factor (TNF)-α-stimulated wild-type mice. Retention of all microbubbles increased (P<0.05) with TNF-α treatment because of increased attachment to activated leukocytes. Extensive attachment of MBp directly to the venular endothelium or to adherent platelet-leukocyte aggregates was observed in TNF-α–stimulated mice, resulting in 4-fold greater (P<0.01) retention of MBp than either MBiso or MB. Enhanced retention of MBp was completely abolished in TNF-α–stimulated P-selectin–deficient mice. The ultrasound signal from microbubbles retained in inflamed tissue was assessed by contrast-enhanced renal ultrasound imaging of the kidneys of mice undergoing ischemia-reperfusion injury. In wild-type mice, this signal was significantly higher (P<0.05) for MBp (12±2 U) than either MBiso (6±3 U) or MB (5±3 U). In P-selectin–deficient mice, the signal for MBp was equivalent to that from control microbubbles.

Conclusions—Microvascular retention of microbubbles targeted to P-selectin produces strong signal enhancement on ultrasound imaging of inflamed tissue. These results suggest that site-targeted microbubbles may be used to assess inflammation, tissue injury, and other endothelial responses noninvasively with ultrasound. (Circulation. 2001;104:2107-2112.)

Key Words: contrast media ▪ cell adhesion molecules ▪ echocardiography ▪ inflammation

We recently demonstrated that tissue inflammation can be assessed noninvasively by ultrasound imaging of microbubbles that are retained by activated leukocytes.1–3 Albumin and lipid microbubbles attach to leukocytes adherent to the venular endothelium and are phagocytosed intact within minutes.1–3 The ultrasound signal from these microbubbles, however, is relatively low because of the small proportion of microbubbles that are retained and viscoelastic damping of microbubbles once phagocytosed.1 This signal has been enhanced by incorporation of specific lipid moieties in the microbubble shell that enhance microbubble avidity for activated leukocytes.2

A more direct method for assessing microvascular inflammatory responses is possible by conjugating ligands for specific endothelial cell adhesion molecules to the microbubble shell.4 Potential advantages of this strategy include a greater number of retained microbubbles, less acoustic damping (because they remain extracellular), and the ability to quantify expression of specific adhesion molecules.

In the present study, we developed lipid microbubbles bearing antibodies against P-selectin, an endothelial cell adhesion molecule expressed during inflammatory responses5 and ischemia-reperfusion.6 We hypothesized that abundant retention of targeted microbubbles in inflamed tissue would result in a strong ultrasound signal and provide a means to image early inflammatory responses by use of intravenous administration of microbubbles. These hypotheses were tested by evaluating the microvascular behavior of targeted microbubbles in wild-type and P-selectin–deficient (P−/−) mice with intravital microscopy and by performing contrast-enhanced renal ultrasound early after ischemia-reperfusion injury.

Methods

Preparation of Microbubbles

Control lipid microbubbles (MB) and microbubbles with monoclonal antibodies against P-selectin (MBp) or isotype control antibodies (MBiso) conjugated to their surfaces were created. Biotinylated
microbubbles containing decafluorobutane gas were prepared as previously described.\(^7\) Approximately \(3 \times 10^6\) biotinylated microbubbles were incubated for 30 minutes with 90 \(\mu\)g streptavidin (Sigma) and washed. Aliquots of the suspension (\(1 \times 10^6\) microbubbles) were incubated for 30 minutes with 75 \(\mu\)g of biotinylated (EZ-Link, Pierce) rat anti-mouse monoclonal IgG1 against P-selectin (RB40.34) or isotype control antibody (R3-34, Pharmingen Inc). The antibody concentration used was determined by flow cytometry experiments (below).

Flow Cytometry

Flow cytometry was used to determine the antibody concentration needed for binding-site saturation. Biotinylated microbubbles (\(1 \times 10^6\)) were incubated as above with streptavidin, then 0.75 to 750 \(\mu\)g of FITC-labeled biotinylated R3-34. Microbubbles (\(1 \times 10^6\) events) were analyzed on a FACSCalibur (Becton Dickinson) to generate histograms of green fluorescence intensity.

Animal Preparation

The study protocol was approved by the Animal Research Committee at the University of Virginia. Mice were anesthetized with an injection (12.5 \(\mu\)L/ip) of a solution containing ketamine hydrochloride (10 \(mg/mL\)), xylazine (1 \(mg/mL\)), and atropine (0.02 \(mg/mL\)). Body temperature was maintained at 37°C with a heating pad. Both jugular veins were cannulated for administration of microbubbles and drugs.

Intravital Microscopy

Inflammation of the cremaster muscle was produced by intrascrotal injections of 0.5 \(\mu\)g murine tumor necrosis factor (TNF-\(\alpha\)) (Sigma) 2 hours before surgery in 5 wild-type mice and in 5 \(P\)-treated wild-type mice obtained from established colonies derived from a gene-targeted founder.\(^6\) Five untreated wild-type mice served as controls. A cremaster muscle was prepared as previously described\(^1\) and was superfused continuously with isothermic bicarbonate-buffered saline. Observations were made with an Axioskop2-FS microscope (Carl Zeiss, Inc) with a saline-immersion objective (SW 40/0.8 numerical aperture). Video recordings were made with a high-resolution CCD camera (C2400, Hamamatsu Photonics) interfaced with a video time-display unit (VTG-33, For-A Ltd) connected to an S-VHS recorder (S95900, JVC).

For microscopy, a fluorescent lipid probe (excitation wavelength \(\approx 500\) nm) was incorporated into the lipid shell.\(^2\) Microscopy was performed with combined fluorescent epi-illumination (460- to 500-nm excitation filter) and low-intensity transillumination. Twenty optical fields encompassing 20- to 40-\(\mu\)m venules were recorded 1 minute after injection of 5 \(\times 10^5\) MB, MB, or MB, microbubbles in random order to determine the number of microbubbles retained and to classify the mechanism of retention as either attachment to leukocytes, attachment to the endothelium, or indeterminate. In 2 of the TNF-\(\alpha\)-treated wild-type mice, injections of MB, were repeated after in vivo labeling of platelets with intravenous injection of 30 \(\mu\)L of 0.05% rhodamine-6G (Molecular Probes).\(^3\) Microscopic observations were repeated with fluorescent epi-illumination with 530- to 560-nm and 460- to 500-nm excitation filters to ascertain the extent of microbubble interactions with activated platelets or leukocyte-platelet complexes.

Venuolar segments were recorded under transillumination at regular intervals. Centerline blood velocities were measured in these segments with a dual-slit photodiode (CircuSoft Instrumentation) and converted to mean blood velocities \((V_c)\) by multiplying by 0.625.\(^10\) Shear rates \((\gamma_c)\) were determined by the equation \(\gamma_c = 2.12(8V_c/d)\), where \(d\) is the vessel diameter and 2.12 is a correction factor for the shape of the velocity profile.\(^11\)

Determination of Leukocyte Adhesion and Rolling Fraction

Intravital microscopy video recordings were analyzed offline. The number of rolling leukocytes \((r_g)\) was determined by counting leukocytes crossing a line perpendicular to the vessel in 1 minute. Leukocyte rolling flux fraction \((F)\), which reflects the percentage of leukocytes in transit that are rolling, was calculated by the equation \(F = r_g / (0.25 \pi d^2 V_c 60 C_L)\), where \(d\) is vessel diameter, \(V_c\) is centerline blood velocity, and \(C_L\) is the systemic blood leukocyte concentration.\(^12\) The number of adhered leukocytes, defined as those stationary for >30 seconds, was expressed per venular surface area.

Contrast-Enhanced Renal Ultrasound

Five wild-type and 3 \(P\)-treated mice were anesthetized, and a kidney was exposed by dorsal paramedian opening of the retroperitoneal space.\(^13\) A hemostatic microvascular clamp (B-IA, ASSI Corp) was placed on the renal pedicle for 30 minutes, then removed. The surgical wound was closed in layers.

Renal ultrasound was performed in the mice undergoing renal ischemia 1 hour after reflow and in 3 control (nonischemic) wild-type mice. Harmonic imaging (Sequoia, Acuson Corp) was performed at a transmission frequency of 2.5 MHz and a mechanical index of 1.9. Images were acquired with a water bath as an interface between the transducer and the dorsum. The acoustic focus was placed at the level of the renal pelvis, and gain settings were optimized and held constant. Data were recorded on a 1.25-cm videotape with an S-VHS recorder (SVO-9500 MD, Sony).

Intravenous injections of 5 \(\times 10^5\) MB, MB, or MB, were made in random order, and ultrasound imaging was not initiated until 8 minutes after each injection to allow clearance of freely circulating microbubbles from the blood pool.\(^2\) The video intensity \((VI)\) in the initial frame was used to determine total tissue concentration of microbubbles within the ultrasound beam (retained and freely circulating).\(^12\) These microbubbles were destroyed by 2 to 3 seconds of continuous imaging (>50 Hz), after which the ultrasound pulsing interval \((PI)\) was increased to 10 seconds to allow complete replenishment of the beam with microbubbles\(^14\) and derivation of signal from freely circulating microbubbles alone.\(^2\) Several averaged precontrast frames were digitally subtracted from the initial contrast-enhanced frame and from several averaged frames obtained at a PI of 10 seconds. Background-subtracted VI was measured from a region of interest placed around each kidney.

Immunohistochemistry

Immunostaining for P-selectin was performed on paraffin-embedded sections of renal tissue. Affinity-purified rabbit polyclonal antibodies against the cytoplasmic domain of P-selectin\(^15\) (kindly provided by Samuel Greene, PhD, University of Virginia) were used as a primary antibody, followed by a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories). Staining was performed with a peroxidase kit (ABC Vectastain Elite, Vector Laboratories) and 3,3'-diaminobenzidine chromagen (Dako). Slides were counterstained with hematoxylin.

Statistical Methods

Data are expressed as mean±SD. Interval comparisons were made with repeated-measures ANOVA. Comparisons of nominal data were made with \(\chi^2\) analysis-of-contingency tables and Yates correction for continuity. Differences were considered significant at a value of \(P<0.05\) (2-sided).

Results

Flow Cytometry

Conjugation of FITC-labeled antibodies to microbubbles was demonstrated by flow cytometry (Figure 1). Incubation of microbubbles with increasing concentrations of antibody resulted in incrementally greater microbubble fluorescent intensity until a saturation concentration of just over 7.5 \(\mu\)g of antibody per 1 \(\times 10^6\) microbubbles was reached (equivalent to an antibody:microbubble ratio of \(3 \times 10^3\):1).
Characterization of Microbubble Retention

The mean blood flow velocity and shear rate in the cremaster venules did not differ between groups (Table). As previously described, TNF-α stimulation in wild-type animals did not affect leukocyte flux fraction but did increase leukocyte adhesion several-fold (Table). Compared with TNF-α–treated wild-type mice, treated P2/2 mice possessed a much lower rolling flux fraction, whereas leukocyte adhesion was minimally reduced (Table).

In the cremaster venules of untreated wild-type mice, retention of MBp was slightly greater than that of MB or MBiso (Figure 2). TNF-α treatment in wild-type mice resulted in a significant increase in retention for all of the microbubble agents, the extent of which was much greater for MBp than control microbubbles (MBiso and MB) (Figure 2). In these animals, retention was greater for MBp than for MB. Enhanced retention of MBp after TNF-α stimulation was completely abolished in P2/2 mice (Figure 2).

Greater retention of MBp than control microbubbles in untreated and TNF-α–treated wild-type mice was largely a result of direct microbubble attachment to the endothelium, which did not occur with the control microbubbles (Figure 3). In TNF-α–treated wild-type mice, the mechanism for MBp attachment was also frequently classified as indeterminate. Enhanced retention of MBp in response to TNF-α was abolished in P2/2 mice because of the absence of endothelial and indeterminate attachment of microbubbles (Figure 3).

### Venular Hemodynamic Parameters and Leukocyte Rolling and Adhesion Data

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type (Untreated)</th>
<th>Wild-Type + TNF-α</th>
<th>P-2/2 + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venular diameter, μm</td>
<td>28 ± 6</td>
<td>29 ± 5</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Blood velocity, μm/s</td>
<td>2055 ± 739</td>
<td>2442 ± 1001</td>
<td>1864 ± 342</td>
</tr>
<tr>
<td>Wall shear rate, s^-1</td>
<td>1251 ± 391</td>
<td>1446 ± 636</td>
<td>1062 ± 214</td>
</tr>
<tr>
<td>Leukocyte rolling flux fraction</td>
<td>0.20 ± 0.24</td>
<td>0.21 ± 0.12</td>
<td>0.07 ± 0.03†</td>
</tr>
<tr>
<td>Leukocyte adherence, mm^-1</td>
<td>203 ± 224</td>
<td>1195 ± 690*</td>
<td>750 ± 384*</td>
</tr>
</tbody>
</table>

*P<0.05 vs wild-type.
†P<0.05 vs wild-type + TNF-α.
Contrast-Enhanced Ultrasound Imaging of Inflammation

Examples of contrast-enhanced renal ultrasound images after ischemia-reperfusion are illustrated in Figure 5. Contrast enhancement in the initial images obtained 8 minutes after injection reflects both retained and freely circulating microbubbles. This signal was much greater for MBp than MB in the wild-type animal undergoing ischemia-reperfusion, whereas signal from MBp was low in the P2/2 mouse. The signal in frames subsequently obtained at a PI of 10 seconds was uniformly very low, indicating the near absence of freely circulating microbubbles.

The mean acoustic signal from retained microbubbles, calculated by subtracting the VI at a PI of 10 seconds from that on the initial frame, was low in control kidneys for all microbubble agents (Figure 6). The signal from retained microbubbles increased for all microbubble agents after ischemia-reperfusion in wild-type mice but was significantly higher for MBp than control microbubbles. Enhanced signal for MBp compared with the other microbubbles in postischemic kidneys was abolished in P2/2 mice. For control microbubbles, there was a trend toward lower signal in P2/2 than in wild-type mice after ischemia-reperfusion.

Endothelial P-selectin expression in glomerular and peritubular vessels by immunohistology was greater in wild-type mice undergoing ischemia-reperfusion than in controls (Figure 7). P-selectin staining of intravascular platelet aggregates, especially in the outer medulla, was occasionally observed only in postischemic kidneys.

Discussion

We recently showed that lipid and albumin microbubbles are retained within the microcirculation of inflamed tissue because of their attachment to activated leukocytes1–3 and can be detected by ultrasound imaging.1 In the present study, we...
These observations, however, do not represent true negative controls, because trauma induced by surgical exteriorization of the cremaster muscle resulted in rapid leukocyte rolling. Early rolling in this setting is almost entirely dependent on recruitment of P-selectin to the surface of venular endothelial cells from preformed stores within Weibel-Palade bodies. Accordingly, a small degree of attachment of MBp directly to the venular endothelium was observed in untreated wild-type animals. There was also infrequent attachment for all 3 agents to the few leukocytes adherent in regions of more severe surgical trauma. For ultrasound experiments, true negative controls were available, because nonischemic kidneys were not instrumented. As a result, there was essentially no ultrasound signal from retained microbubbles in control kidneys during ultrasound imaging.

More intense inflammation in the cremaster muscle was produced by cytokine stimulation with TNF-α. Cytokine stimulation of the cremaster muscles resulted in a marked increase in venular leukocyte adherence. Consequently, the extent of microbubble attachment to leukocytes increased. The degree of leukocyte retention was slightly greater for microbubbles that contained antibodies (either control or P-selectin-specific antibody), most likely because of enhanced opsonization or interaction with immunoglobulin receptors expressed by leukocytes. Leukocyte recruitment in the cremasteric microcirculation after treatment with TNF-α is promoted by increased venular endothelial expression of P-selectin. Hence, retention of MBp was much greater than for either of the control microbubbles, largely because of a population that attached directly to the venular endothelial surface.

Early inflammatory responses after ischemia-reperfusion of the heart and kidney are supported by marked luminal expression of P-selectin. For ultrasound imaging studies, a model of renal ischemic injury was used that results in reperfusion is blunted, which probably explains the lower ultrasound signal from retained microbubbles in postischemic kidneys during ultrasound imaging.

Figure 7. Immunohistochemistry of control and postischemic kidneys in wild-type mice. In postischemic kidneys, P-selectin expression in glomerular and peritubular vessels (A) was greater. In the outer medulla (B), large vascular platelet aggregates (arrows) were seen only in postischemic kidneys. Bar=50 μm.

Accumulation of leukocytes in regions of inflammation relies on a sequence of events characterized by leukocyte capture and rolling along the venular endothelium, progressive activation of rolling leukocytes, firm adhesion, and finally transmigration through the vessel wall. The initial process of leukocyte rolling is mediated primarily by the selectin family of adhesion molecules. In the present study, we targeted microbubbles against P-selectin by conjugating monoclonal antibodies against murine P-selectin to the surface of lipid-shelled microbubbles via a biotin-streptavidin system.

Retention of P-selectin–targeted microbubbles in inflamed tissue was evaluated by direct microscopic observation of their behavior in the cremasteric microcirculation of mice and by detection of microbubble signal after renal injury. For intravital microscopy experiments, untreated wild-type animals were used to evaluate microbubble behavior at baseline.

Figure 8. Mechanisms of microbubble retention. Nontargeted and probably targeted phospholipid microbubbles bind to adherent leukocytes through complement (C'). Microbubbles conjugated with antibody (MB, or MBiso) show slightly enhanced binding, most likely due to interactions with immunoglobulin receptors on leukocytes (FcR). Only microbubbles conjugated with mAb RB40.34 against P-selectin (MBp) also bind to P-selectin on endothelial cells (EC) or platelets (Plt).
Activated platelets also express P-selectin, which can mediate their attachment to activated leukocytes after ischemia-reperfusion injury.\textsuperscript{24} We investigated whether platelet bridging between microbubbles and leukocytes could at least partially account for MB\textsubscript{r} retention that occurred by an “indeterminate” mechanism in TNF-\textalpha- treated wild-type mice. In vivo fluorescent labeling of platelets confirmed attachment of MB\textsubscript{r} microbubbles to platelet-leukocyte aggregates adherent to the venular endothelium, whereas attachment to platelets that transiently adhered directly to the endothelium was not seen. In the renal ischemia-reperfusion model, immunohistology suggested that cellular aggregates containing platelets may also have contributed to higher signal intensity for retained MB\textsubscript{r} microbubbles.

The rationale for developing microbubbles targeted against P-selectin was to increase microbubble retention within the microcirculation, and hence, signal on ultrasound imaging of inflamed tissue. Although other acoustically active contrast agents with antibodies against intercellular adhesion molecule-1 conjugated to their surface have been developed,\textsuperscript{4,25} the clinical feasibility of imaging with these agents has not been demonstrated. Our results indicate that ultrasound imaging of targeted microbubbles may provide a means to noninvasively assess inflammation and tissue injury. It must be acknowledged that tissue accumulation of microbubbles directed toward a molecular target is also likely to be influenced by other physiological parameters, such as regional perfusion. Therefore, further investigation into how microbubble retention is influenced by tissue hyperemia or postischemic microvascular “no-reflow” is warranted. Variation of clearance from the vascular pool for different microbubble agents, depending on their composition, may also affect the extent of microbubble retention by limiting or enhancing their recirculation through the target organ.

The results of this study imply that contrast ultrasound with targeted microbubbles not only may be useful for assessing inflammation but also could potentially be used to image other endothelial phenotypes (such as angiogenesis, tumor formation, and metastasis). Moreover, recent reports of enhanced gene transfer\textsuperscript{26,27} in vivo by use of acoustic disruption of microbubbles suggest that the potential applications of targeted microbubbles may extend beyond diagnostic studies and into the realm of therapeutics by site-specific delivery of drugs or genes.

Acknowledgments

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References


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