Background and Purpose—Targeted ultrasound contrast agents have recently been developed to adhere selectively to specific pathogenic materials such as plaque or thrombus. Administration of such microbubbles has potential to aid transcranial Doppler ultrasound (TCD) detection of emboli and to act as markers for distinguishing one embolic material from another. The purpose of this study was to investigate whether TCD detection of circulating thrombus emboli would be enhanced by the addition of targeted microbubbles.

Methods—Binding of microbubbles to the surface of the thrombus was confirmed by scanning electron microscopy. Targeted and control bubbles were then introduced to thrombus and tissue-mimicking material circulated under pulsatile-flow conditions in an in vitro flow rig. Embolic signal intensities before and after introduction of the bubbles were measured by TCD.

Results—Targeted microbubbles enhanced TCD signal intensities from thrombus emboli by up to 13 dB. The bubbles were capable of binding to moving thrombus when injected into the flow circuit in low concentrations (≈36 bubbles per 100 mL) and were retained on the thrombus under pulsatile-flow conditions. Signal intensities from similarly sized pieces of tissue-mimicking material were not enhanced by injection of targeted bubbles.

Conclusions—Injection of appropriately targeted microbubbles significantly enhances TCD detection of circulating thrombus emboli in vitro. (Stroke. 2007;38:2726-2732.)

Key Words: atherosclerosis  ■  embolism  ■  microbubbles  ■  thrombus  ■  ultrasonics
antibody fragment–grafted microbubbles (immunobubbles). Injection of immunobubbles has potential to enhance detection of weakly scattering emboli owing to an increase in backscattered ultrasound power produced by the attached bubbles. Detection of an increase in embolic signal intensity has potential to provide a means of distinguishing one embolic material from another. Microbubbles also have a number of other promising potential applications, such as encapsulation of “clot-busting” pharmaceuticals and enhanced embolus destruction by sonothrombolysis.

In the present study, we tested abciximab-grafted microbubbles designed to attach to activated platelets on the surface of thrombus. We confirmed binding of the bubbles by scanning electron microscopy (SEM) and TCD. Experiments simulating the flow of blood and emboli through an artery showed significant enhancement of Doppler embolic signal intensities after introduction of targeted microbubbles.

**Materials and Methods**

**Microbubble Preparation**

The bubbles tested during this study were abciximab immunobubbles developed by Bracco Research SA (Geneva, Switzerland). The bubbles typically ranged between 1 and 4 μm and consisted of decafluorobutane encapsulated within a lipid shell. The targeted bubbles had antibody fragments grafted onto their membranes that specifically targeted the glycoprotein IIb-IIIa receptor present on the surface of platelets. These microbubbles were obtained by addition of the thiol-activated antibody fragment to maleimide-functionalized phospholipids incorporated into the shell. Control microbubbles were obtained in the same way but with a nonspecific human antibody fragment. Targeted and control microbubbles were provided in the form of 100 mg dry lyophilized powder and stored in glass vials containing an atmosphere of decafluorobutane. The bubbles were reconstituted by vortexing with 5 mL phosphate-buffered saline. This suspension was centrifuged, the infranatant was discarded, and the bubbles were resuspended in an additional 5 mL of fresh saline. Bubble concentrations were estimated on the basis of an original bubble count of 1.8×10⁸ bubbles per vial when reconstituted in 1 mL saline. The in vial half-life of the bubbles was ~3 hours.

**Embolus Preparation**

Clinically realistic thrombi were generated by the Chandler loop method. Thrombi produced in the Chandler loop closely resemble arterial thrombi formed in vivo, with a platelet-rich “head” and a fibrin-rich “tail.” Freshly prepared thrombi were cut into small pieces and measured with a traveling optical microscope equipped with a Vernier scale capable of ±0.01-mm accuracy. As a control, similarly sized “emboli” were also cut from pieces of an agar-based tissue-mimicking material.

**Scanning Electron Microscopy**

To prepare the samples for SEM, fresh thrombi of approximately equal size were exposed for ~3 seconds to a ~3.6×10⁸ bubbles/mL suspension of either the targeted or control microbubbles. The bubble-thrombus complex was gently washed in saline and transferred via pipette to a mixture of HEPES buffer and 25% glutaraldehyde solution. Samples were then dehydrated through a series of ethanol concentrations increasing from 50% to 100% and dried with LCO₂ with a Bal-Tec CPD-030 critical-point drier. SEM images were obtained with a field-emission gun SEM (FEI Sirion 200, FEI Electron Optics, Eindhoven, Netherlands).

**Figure 1.** Closed-loop flow rig designed to evaluate the mean Doppler signal intensity of circulating emboli. Emboli and microbubbles were introduced to the flow rig through the funnel reservoir. Pulsatile flow was generated with a peristaltic pump. MCMG indicates multichannel multigate.

**Flow-Rig Experiments**

Flow-rig experiments were conducted with a closed-loop flow phantom (Figure 1). The circuit was constructed of C-flex tubing with a 4.6-mm internal diameter and a 0.8-mm wall thickness (Cole-Parmer, London, UK). Pulsatile flow, with a peak “systolic” velocity of ~30 cm·s⁻¹, was produced by a peristaltic pump (HR flow-inducer type 2000, Watson Marlow, Cornwall, UK). The circulating fluid consisted of 100 mL of 0.9% saline mixed with ~2 mL blood-mimicking fluid.

The Doppler system used in this study was a noncommercial multigate TCD device with exceptionally high dynamic range. System settings were similar to those of a commercial TC22 TCD machine (SciMed, Bristol, UK), but with a lower thermal index and pulse-pressure amplitude and a similar signal-to-noise ratio. The artificial vessel was imsonated with a nominal 10-mm sample length at a depth of 50 mm and an insonation angle of ~30°. Recorded data were saved directly to hard disk for later analysis.

Because the circulating fluid was translucent, passage of an embolus through the sample volume could be verified by visual inspection. Embolic signals were accepted for analysis only when their times of appearance in the sonogram matched those visually recorded by an observer. Analyses of the recordings were performed with an in-house embolic signal analysis program to replay the sonogram and audio signal in real time. Embolic signal intensities were measured relative to the background signal with a measured embolus-to-background ratio (MEBR) in decibels. All of the analyzed emboli had properties that were consistent with international embolus detection criteria.

**Thrombi Immersed in Microbubbles**

After prior circulation of the fluid to remove air from the system, single pieces of thrombus of known size were introduced to a microbubble-free circuit via the funnel reservoir (Figure 1). Each piece of thrombus circulated past the transducer 10 times, thus generating 10 MEBR values. Optical microscopy was performed before and after circulation to confirm that no fragmentation had occurred. If the thrombus was still intact, it underwent an ~3-second exposure to a ~3.6×10⁸ bubble/mL suspension of either the targeted or the control bubbles. The bubble-thrombus complex was gently washed to remove any unbound bubbles and reintroduced to the flow phantom. The thrombus-bubble complex was then allowed to circulate another 10 times in the same circulating fluid, and the new average MEBR and SD were estimated. Finally, the embolus dimensions were measured again. Fragmentation was apparent in ~1 of 4 emboli, which were not included in our analysis.

**Microbubbles Injected Into the Flow Circuit**

In the last set of experiments, thrombi were circulated 10 times, and then 0.1 mL of a dilute (~360 bubbles/mL) suspension was injected...
into the funnel reservoir (Figure 1). Once introduced to the flow circuit, the dilution of bubbles was sufficiently low that discrete signals from the passage of individual bubbles through the sample volume could be observed. Doppler signals from the circulating thrombus-bubble complex were distinguishable from those of free circulating microbubbles by their higher intensity and longer duration. The time taken to complete 1 circuit was \( \frac{1}{60} \) seconds, with a total duration for 10 circulations varying between 8 and 10 minutes. After the embolus had circulated another 10 times, average MEBR values before and after injection of the bubbles were compared. In calculating the intensity of the thrombus-bubble complex, we made every effort to ensure that there were no free microbubble signals in the background window used for calculation of MEBR. All experiments were performed within 20 minutes, which is considerably shorter than the \( \sim 3 \)-hour half-life of the bubbles.

Statistics
Mean MEBRs and SDs were estimated from an average of at least 10 measurements. The difference in MEBR before and after introduction of bubbles (\( \Delta \text{MEBR} = \text{MEBR}_{\text{after}} - \text{MEBR}_{\text{before}} \)) was assumed to have an SE of \( SE = \sqrt{\frac{\text{SD}_{\text{before}}^2}{n} + \frac{\text{SD}_{\text{after}}^2}{n}} \). The a priori hypothesis that microbubbles would produce an enhancement in MEBR was tested with a 1-tailed Mann–Whitney \( U \) test. Nominal significance levels (\( \alpha \)) are stated in the text. The level of correlation of MEBR with time was characterized by a line-of-regression analysis, estimation of an \( R^2 \) value, correlation coefficient (\( r \)), and significance level \( P \). The symbol \( \Delta \text{MEBR} \) denotes the mean increase in MEBR averaged for several samples.

Results
Microscopic Investigation of Binding
SEM images were obtained for an unexposed thrombus and for thrombi that had been exposed to either the control bubbles (Figure 2) or targeted bubbles (Figure 3). All of the images showed a mesh of fibrin and several areas rich in platelets (Figure 2a). Some images also showed activated platelets (Figure 2b) and occasional white blood cells (Figure 3c). Images of thrombi exposed to control bubbles showed a small number of nonspherical objects of \( \sim 3-\mu m \) diameter, some of which resemble “squashed” or decomposing microbubbles (Figure 2c). In thrombi not exposed to any form of bubble, no bubblelike structures were observed.

Microscopy images for thrombi exposed to targeted bubbles clearly showed attachment of intact, targeted microbubbles to the thrombus (Figure 3). Bubbles appeared as \( \sim 1-4-\mu m \) spherical structures, which were typically larger and more spherical than platelets. Microbubbles can be distinguished from white blood cells because of the smoothness of the bubble surface (Figure 3c).

Thrombi Immersed in Microbubbles
Immersion of thrombi (with mean diameters of 1.5, 2.1, and 2.5 mm) in a concentrated solution of targeted bubbles produced an average increase in mean MEBR of \( \sim 13 \) dB (the Table). Representative data from 2 of these thrombi are shown in Figure 4a. Increases in MEBR were of high statistical significance, based on a 1-tailed Mann–Whitney \( U \) test (\( \alpha = 0.0005 \)). Variations in MEBR over time were investigated by determining the gradient of the line of regression for consecutive measurements of the same embolus and were not found to be statistically significant. For targeted bubbles, enhancement of embolic signal intensity persisted under pulsatile-flow conditions and did not diminish during the 8 to 10 minutes required for 10 circulations. This suggests that bound, targeted bubbles were robust to pulsatile-flow conditions.
As a control, 3 similar sizes of thrombi (1.5, 2.15, and 2.3 mm) underwent the same process but were instead bathed in a suspension containing control bubbles (the Table). Representative data from 2 thrombi are shown in Figure 4b. Initial exposure to control bubbles appeared to increase MEBRs by a similar amount as for targeted bubbles. However, the enhancement from control bubbles was short-lived (<8 minutes) and decreased with time in the circuit. All 3 thrombi exposed to control bubbles exhibited a statistically significant negative correlation between MEBR and the times of consecutive measurements ($r = -0.9$, $P<0.001$). This suggests that “binding” of control bubbles did not persist under pulsatile-flow conditions. Gradual detachment of control bubbles reduced the increase in MEBR, averaged for 10 cycles, to $\sim 8$ dB.

With a null hypothesis stating that “there was no change in MEBR before and after introduction of the bubbles,” the control microbubbles produced a statistically significant increase in MEBR in 2 of the 3 thrombi. For these thrombi, the null hypothesis was rejected with a level of significance of $\alpha=0.0005$ (1.5-mm thrombus) and $\alpha=0.001$ (2.3-mm thrombus; Figure 4b). For the third thrombus (2.15 mm), the null hypothesis was accepted, indicating that there was no statistically significant increase in MEBR.

**Microbubbles Injected Into the Flow Circuit**

To mimic injection of microbubbles under pulsatile-flow conditions, measurements were performed for 4 sizes of thrombi (with mean diameters of 0.7, 1, 1.7, and 1.9 mm) before and after injecting 0.1 mL of a dilute suspension containing $\sim 360$ microbubbles/mL into the flow circuit. The targeted bubbles produced a clear enhancement in MEBRs (the Table). Representative data are shown in Figure 5a. Levels of significance were $\alpha=0.001$ for the 0.7-mm thrombus and $\alpha=0.0005$ for the other 3 sizes (1, 1.7, and 1.9 mm).

The experiment was repeated with control bubbles for 3 sizes of thrombus (1.1, 1.5, and 1.8 mm), but no statistically significant increase in MEBR for any of these thrombi was observed (the Table and Figure 5b). To confirm active binding to the thrombus, we also tested for attachment of targeted microbubbles to synthetic emboli composed of tissue-mimicking material. These tissue-mimicking material emboli do not contain glycoprotein IIb-IIIa receptors. No statistically significant increase in MEBR for tissue-mimicking material was observed.

**Discussion**

This study confirms previous investigations showing target-specific binding of microbubbles to static thrombus. Our SEM images provide direct evidence that targeted abciximab microbubbles attach to the surface of thrombus. These images complement those obtained by Tsutsui et al., which showed binding of bubbles designed to attach to damaged endothelium.

Our investigation differs from previous reports by using TCD to demonstrate binding of targeted microbubbles to circulating thrombus emboli. To test whether bubbles were capable of attaching to moving thrombus under pulsatile-flow conditions, we injected dilute solutions of targeted and control microbubbles.
into the flow circuit. This experiment was designed to verify whether a dilute solution of microbubbles injected upstream of the source of embolization would be capable of successfully adhering to moving thrombus emboli in flowing blood. We observed a clear difference between the injection of targeted and control bubbles: thrombi introduced to targeted bubbles were significantly enhanced ($\Delta$MEBR = 8.2 dB; the Table), whereas signals from thrombi receiving the control bubbles were not ($\Delta$MEBR = 1.4 dB; the Table). We concluded that only targeted bubbles were capable of attaching to and enhancing the MEBRs of moving thrombus under clinically realistic flow conditions.

The advantages of ultrasound contrast agents for enhancement of conventional ultrasound images are well known but have not yet been applied to TCD embolus detection. Thromboemboli have a density similar to that of blood, and are therefore, the most difficult composition of embolus to detect with TCD. A major benefit of targeted microbubbles would be enhancement of the detection of weak embolic signals.

One limitation of our study was that the sizes of thrombi used in these experiments were larger than expected in vivo. Doppler signal enhancement of smaller emboli may not be as marked as described in this study. Another limitation is that

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†Mean MEBR increase and SD for thrombus incubated with microbubbles before being added to the flow circuit.
‡Injection of a dilute solution of microbubbles to precirculating thrombus.
§Injection of a dilute solution of microbubbles to precirculating tissue-mimicking material. Underneath each column, the overall average increase in MEBR (denoted by $\Delta$MEBR) and SD are shown.

Figure 4. a, Exposure of thrombus to a solution of targeted microbubbles enhanced MEBRs to a higher level (filled squares) than measured from the thrombus alone (open circles). The x axis shows time from the start of the experiment. Lines of regression (with associated $R^2$ values) show no significant variation in MEBR with time. b, Control bubbles initially enhanced MEBR; however, the lines of regression show that this enhancement declined with time ($r=0.9, P<0.001$).
free bubbles in the circuit continued to circulate rather than be expelled. This prevented us from studying the dose dependence of the enhancement, as large numbers of free microbubbles in the fluid would have enhanced the background signal and reduced the MEBR. In clinical practice, any unbound bubbles would be flushed from the circulation, so the optimum concentration in vivo would be different from that determined in vitro.

Brief exposure of thrombi to a concentrated solution of targeted bubbles produced an average rise in MEBR of $13.1 \pm 5$ dB (the Table). Despite the pressure cycles of the pump, ultrasound insonation, fluid flow forces, diffusion of gas from the bubbles, and bubbles becoming trapped in the reservoir, targeted bubbles remained bound to the thrombus for the duration of our measurements. No significant decrease in MEBR with time between consecutive circulations over 10 minutes was observed (Figure 4a). Increases in MEBRs of $8$ dB were observed for doses of targeted microbubbles as low as $36$ microbubbles in $100$ mL, which indicates a high affinity between the targeted bubbles and thrombus. Concentrated control bubbles produced an increase in MEBR of $7.9 \pm 3.7$ dB, but there was a significant decrease in MEBR with time (Figure 4b). This implied that control bubbles were “washed away” by flow forces in the circuit. When control bubbles were injected into the flow circuit in dilute concentrations, no MEBR enhancement was observed.

Administration of immunobubbles to patients with suspected “unstable” carotid or cardiac plaques could greatly enhance ultrasound images and lead to improved assessment of plaque stability and embolus composition. Although free bubbles would be rapidly expelled from the circulation, we have demonstrated that bound bubbles are robust to physiologic flow conditions and have the potential to survive in the body for longer durations, allowing ultrasound imaging or embolus detection to be performed. In prolonged TCD monitoring of patients with carotid stenosis, emboli are extremely infrequent, and either bubbles with longer lifetimes or a regular “top up” may be required. Bubbles of increased longevity are currently under development.

During TCD embolus detection, an increase in average MEBR after injection of immunobubbles could indicate that a large proportion of emboli were of either thrombus or platelet origin. Microbubbles may therefore be potentially useful for distinguishing between different embolic materials, especially if various, more specific targeting moieties are used. To verify that immunobubbles examined in the current study were target-specific, experiments were performed with thrombus-targeted bubbles injected into a circuit containing synthetic tissue-mimicking material. No enhancement of signal from tissue-mimicking material emboli was observed ($0.0 \pm 0.3$ dB). However, because plaque surfaces and plaque debris can be thrombogenic and contain superimposed thrombus, immunobubbles may be unable to distinguish atheromatous from purely thrombotic embolic materials in vivo. The bubbles investigated herein adhered to activated platelets, but there is a range of potential target-
ing antibodies that could selectively bind to other pathogenic materials and might not suffer from the same attraction to superimposed thrombus as thrombus-targeted bubbles. The potential for distinguishing between pathogenic materials needs to be investigated in a more physiologically and hematologically realistic study.

The possibility that microbubbles could produce false-positive signals by binding to other material such as platelets or platelet microparticles was also considered. Single platelets or platelet microparticles would be tiny compared with the immuno-nobubbles and therefore, result in an ultrasound signal that would be similar to that from a free circulating bubble.

Last, we considered the possibility that microbubbles could induce a pharmacologic antiplaetlet effect. The c7E3 antibody (abciximab) is used therapeutically to prevent the binding of fibrinogen to platelets. However, to inhibit platelet aggregation, there needs to be >70% receptor occupancy of the drug (ie, >70% of ≅50 000 glycoprotein IIb-IIIa receptors on the platelet have to be occupied). The ratio of microbubbles to platelets would be ≅1:109, which would not significantly inhibit platelet aggregation.

In conclusion, we confirm that targeted microbubbles are capable of selectively binding to moving thromboemboli under pulsatile-flow conditions. Targeted microbubbles produce pronounced enhancements in Doppler signals from thrombus but not from tissue-mimicking material. We therefore propose that targeted microbubbles could form a new basis for enhanced target-specific detection of emboli by TCD.

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Disclosures

None.

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