Molecular Imaging of Human Thrombus With Novel Abciximab Immunobubbles and Ultrasound

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Background and Purpose—Molecular imaging of therapeutic interventions with targeted agents that simultaneously carry drugs or genes for local delivery is appealing. We investigated the ability of a novel microbubble carrier (immunobubble) for abciximab, a glycoprotein IIb/IIIa receptor inhibitor, for ultrasonographic molecular imaging of human clots.

Methods—Human thrombi were incubated with immunobubbles conjugated with abciximab. Control clots were incubated in either saline or with immunobubbles conjugated with nonspecific antibody. We evaluated immunobubble suspensions with variable concentrations of encapsulated gas and measured mean acoustic intensity of the incubated clots. In vivo molecular imaging of human thrombi with abciximab immunobubbles was evaluated in a rat model of carotid artery occlusion.

Results—Mean acoustic intensity was significantly higher for abciximab immunobubbles as compared with control immunobubbles under all conditions tested with maximum difference in intensity at a gas volume of 0.2 μL (P=0.0013 for mechanical index 0.05, P=0.0001 for mechanical index 0.7). Binding of abciximab immunobubbles to clots in vitro led to enhanced echogenicity dependent on bubble concentration. In vivo ultrasonic detectability of carotid thrombi was significantly higher for clots targeted with abciximab immunobubbles (P<0.05). Quantification of in vivo contrast enhancement displayed a highly significant increment for abciximab immunobubble-targeted clots compared with nonspecific immunobubble-targeted clots (P<0.0001) and to native clots (P<0.0001).

Conclusions—This study demonstrates the feasibility of using a therapeutic agent for selective targeting in vascular imaging. Abciximab immunobubbles improve visualization of human clots both in vitro and in an in vivo model of acute arterial thrombotic occlusion. (Stroke. 2007;38:1508-1514.)

Key Words: abciximab ■ carotid ■ microbubble ■ molecular imaging ■ stroke ■ ultrasound

Molecular imaging is a rapidly evolving discipline with the goal of developing tools to display and quantify molecular and cellular targets in vivo. Recent advances have allowed in vivo sensing of inflammation, apoptosis, and gene expression. Particularly appealing is the idea of developing biologic imaging sensors that serve not only as detectors, but also as carriers of drugs or genes. Such agents would allow simultaneous diagnosis, targeted therapy, and monitoring of disease with a single agent, eg, imaging of thrombosis, targeted delivery of thrombolytic therapy, and monitoring of thrombus dissolution. Ultrasound contrast agents displaying high acoustic impedance mismatch and incorporating targeting ligands onto their surface are particularly relevant for molecular imaging. In their first report on targetable ultrasonic contrast agents, Lanza et al demonstrated an increment in ultrasonic echogenicity of porcine and canine thrombi using antifibrin-targeted microemulsions. Newly developed microbubbles bearing bioconjugate ligands to the glycoprotein IIb/IIIa receptor have been shown to bind specifically to platelets. An increment in microbubble adherence to human vascular thrombi in an in vitro model and improved visualization of vena cava and atrial thrombi in dogs have been demonstrated for receptor selective targeted microbubbles. Evidence for effective echographic thrombus visualization with human platelet-targeted microbubbles under in vivo conditions is lacking, however, and there are no studies of imaging-targeted microbubbles using an in vivo model of arterial occlusion.

In this study, we evaluate the potential of binding abciximab, a monoclonal antibody against the glycoprotein IIb/IIIa receptor, to the shell surface of microbubbles for ultrasonographic molecular imaging of acute arterial thrombotic occlusion.

Methods

Immunobubble Description and Characterization

Microbubbles targeted to human platelets (immunobubbles) were developed using abciximab (ReoPro injectable solution), an antibody fragment specific for the GPIIb/IIIa receptor expressed by activated
platelets. Briefly, the immunobubbles are based on a monolayer of phospholipids in which a maleimido-4(p-phenylbutyrate)-bearing phospholipid is incorporated for reaction with the thiol-activated abciximab. Control immunobubbles conjugated with a structurally similar, but nonspecific antibody, were developed following the same procedure by grafting a nonspecific human Fab (Bethyl #P80–215).

After preparation for use, one vial yielded approximately 2 × 10^6 immunobubbles as measured by electrophore sensing using a Coulter counter (Multisizer 3; Beckman-Coulter). The immunobubbles had a number average diameter of 1.5±0.2 μm and a volume average diameter of 2.8±0.4 μm with a volume fraction of bubbles with sizes above 8 μm of less than 5%. Before coupling, part of the abiciximab was iodinated by the Iodogen method using Na125I. This enabled determination of the number of abiciximab molecules that were present at the surface of the immunobubbles (16 500 molecules) resulting in approximately 2000 molecules per micron squared.

**Static Binding on Platelets**

Platelet-coated 24-well plates were prepared by scaling up an established protocol using washed human platelets isolated by differential centrifugation from heparinized human whole blood collected from healthy volunteers. The platelet-coated central well of each plate was filled with a solution of 50 vol% normal human serum collected from healthy volunteers. The platelet-coated central well of plate was then sealed, turned upside down, and incubated for 30 minutes.

After incubation, the immunobubble suspension was discarded and the platelet-coated wells were rinsed 3 times with phosphate-buffered saline. Micrographs were captured with a digital camera mounted on an inverted microscope for comparison of static binding between abiciximab and nonspecific control immunobubbles.

**Characterization of the Effect of Bubble Gas Volume on Contrast Enhancement**

To evaluate the effect of different microbubble gas volumes on contrast enhancement of human clots in vitro with abciximab and nonspecific immunobubbles, 50 μL of CaCl2 solution 100 mmol/L, 50 μL of human thrombin solution 50 U/ml, and 400 μL of citrated blood from healthy volunteers were mixed in 24-well cell culture plates. After coagulation and maturation at room temperature overnight, the clots were cut into pieces measuring 2 × 10 mm. The thrombi were then incubated together with abiciximab immunobubbles or nonspecific immunobubbles (the adequate amount of bubble suspension was introduced in 800 μL of phosphate-buffered saline with 50% plasma to obtain the desired total microbubble gas volumes, ranging from 0.02 μL to 1.6 μL) for 30 minutes and placed on a rotating wheel. Two washing steps with 800 μL of saline were performed before visualization to avoid nonspecific microbubble binding.

Thrombi were placed on a Sonar Air pad in a saline-filled box. A specific contrast imaging modality, pulse inversion harmonic imaging, was used to image the clots (Philips HDI 5000, L7-4 linear probe). For each thrombus, digital images at baseline and maximal intensity of the clot were measured after incubation, and the baseline intensity. In all the captured frames, an area of interest was drawn on the thrombus. The mean acoustic intensity of this area was calculated from a calibration curve and was expressed as a function of incubated bubble volume. Three clots were used to obtain the mean and standard deviation for each condition. Results of these experiments were used to determine the optimal bubble concentrations for further in vitro and in vivo experiments (see subsequently).

**In Vitro Visualization of Human Clots With High-Resolution Compound B-Mode Imaging**

For in vitro and in vivo evaluation of high-resolution B-mode imaging of thrombus enhancement after incubation with abciximab or nonspecific immunobubbles, blood clots were obtained by spontaneous coagulation of whole human blood samples from healthy volunteers. Blood was drawn into Vacutainer tubes with kaolin-coated globules to induce thrombotic activity and injected into a polyelectrolyte tube with an internal diameter of 1 mm where it was allowed to coagulate for 2 hours. Afterward, clot material was removed by saline flushing and cut into 4-mm pieces. Clots were incubated in vitro in a 1 mL suspension of platelet-targeted immunobubbles and control clots in nonspecific immunobubbles or saline 0.9%, respectively, as described previously. A following washing step in saline was performed as described previously.

The human clots were transferred to petri dishes containing normal saline. High-resolution B-mode imaging was performed using a Philips HDI 5000 platform with a 7 to 15 MHz dynamic range linear transducer (L 15 to 7, MI 0.05) in compound imaging mode using the same parameters as in the animal model of arterial occlusion (see subsequently). Mean linear acoustic intensity of the clots was determined with HDILab, a software quantification tool provided by Philips that operates on unprocessed ultrasound signals. To facilitate graphic illustration of large differences in acoustic intensity, computed mean linear values were converted to decibels.

Mean group values were compared by univariate analysis of variance and one-sided, nonpaired Student t test (SAS). Values of P<0.05 were considered significant for analysis of variance; significance level for Student t test was set to P<0.05. When multiple testing was performed, level of significance was adjusted following Bonferroni correction.

Human clots were obtained as mentioned previously by spontaneous coagulation of whole human blood samples from healthy volunteers. Blood was drawn into Vacutainer tubes with kaolin-coated globules and allowed to coagulate for 2 hours. Afterward, clot material was removed by saline flushing. Clots were incubated in vitro in a 5 mL suspension of platelet-targeted immunobubbles and a washing step in saline was performed as described previously. Clots were then imaged in saline at room temperature with a specific contrast agent imaging mode (power modulation and pulse inversion harmonic imaging) at low MI (0.05) using a linear transducer (L9–3) on a Philips IU22 platform. The clots were then ionopped with a high MI for 30 seconds to destroy all attached immunobubbles. This ability to destroy bubbles with a high MI has been demonstrated in previous studies. Another image was taken of the clot directly after bubble destruction. The clot was then reincubated in abciximab immunobubbles as described previously, washed in saline, and imaged again in the saline bath using exactly the same ultrasound parameters. Acoustic intensity of the clot was measured after incubation, bubble destruction, and reincubation.

**Animal Model of Arterial Occlusion With Human Clot**

All experiments were approved by the local government authorities in accordance with the animal protection guidelines. Wistar rats (N=19) weighing 330 to 580 g were anesthetized with 1.5% isoflurane. During the experiment, normal body temperature was maintained with a heating pad. After surgical exposure of the right carotid artery system, the internal carotid artery was ligated. A 4-mm partially occlusive human clot was introduced into the distal common carotid artery over an external carotid artery polyelectrolyte tube catheter with an internal diameter of 0.57 mm. The external carotid artery was then ligated. Seven rats received a human clot preincubated with abciximab-targeted immunobubbles, 7 rats received a clot preincubated in control immunobubbles, and 5 rats received a native clot.
Transcutaneous ultrasound was performed using the same parameters as in the in vitro visualization studies (see previously). Ultrasonic images were obtained immediately after thrombus insertion with a low MI (0.05) to avoid microbubble destruction. The clots were then scanned with a high MI (1.3) to destroy the bubbles that were attached to thrombus through preincubation, either abciximab- or nonspecific immunobubbles. Low MI (0.05) was used to document the loss in acoustic intensity after bubble destruction. Detectability of preincubated intraluminal clots was scored as either detectable with high or low contrast enhancement or undetectable. The $\chi^2$ test was used to compare frequency distributions of thrombus scores. For statistical analyses, differences in pixel gray-scale values assessed in freeze frame images of in vivo clots were analyzed and compared by analysis of variance and Student $t$ test.

To determine whether adequate visualization of thrombus could be achieved during intravenous application of microbubbles in this animal model of arterial occlusion, serial ultrasound examinations at low MI (0.05) were performed during constant administration (through a left femoral vein catheter at the rate of 0.2 mL/min) of targeted immunobubbles, nontargeted microbubbles, or saline. The 7 rats with a human thrombus preincubated in targeted immunobubbles each received a 5 mL suspension of targeted immunobubbles; the animals of the 2 control groups (seven rats with a human clot preincubated in nonspecific microbubble suspension, 5 rats with a non-treated native human clot) each received a 5 mL suspension of nonspecific immunobubbles or saline, respectively. Afterward, the animals were decapitated in deep anesthesia; the right carotid artery system was preserved and presence of thrombus was confirmed histologically.

**Results**

**In Vitro Static Binding on Platelets**

As shown in Figure 1, the microscopic examination of platelet deposits after static incubation with either the nonspecific Hu-Fab immunobubbles or the abciximab immunobubbles clearly demonstrates that the targeted immunobubbles firmly bind to platelets. There is negligible binding of nonspecific immunobubbles.

**Effect of Bubble Gas Volume on Contrast Enhancement**

To explore the relationship between contrast enhancement and bubble dose, we examined a series of clots targeted with different concentrations of microbubbles corresponding to different amounts of total encapsulated gas volumes using pulse inversion harmonic imaging. As shown in Figure 2, the maximum intensity expressed as power/area strongly increased with increasing bubble concentration. At an encapsulated gas volume of 0.8 $\mu$L, both specific and nonspecific binding of microbubbles were highest, independent of the MI used. However, absolute values for higher gas volumes were approximately 50% higher when operating with a MI of 0.7 (Figure 2b) compared with 0.05 (Figure 2a). The maximum difference in intensity comparing specific with nonspecific microbubble binding was reached at a gas volume of 0.2 $\mu$L both for low and high MIs, indicating that the highest specificity in ultrasonic imaging is achieved using microbubbles with an encapsulated gas volume of 0.2 $\mu$L ($P=0.0013$ for MI 0.05, $P=0.0001$ for MI 0.7, see Figure 2). This volume was used in further in vitro and in vivo experiments.

**In Vitro High-Resolution Compound B-Mode Imaging**

In a further series of experiments, we examined whether contrast enhancement could be documented with high-resolution B-mode imaging in vitro using diagnostic frequencies required for visualization of small thrombi (1 $\times$ 1 $\times$ 4 mm) in the in vivo rat model of carotid artery occlusion. All clots, including the nonpreincubated control clots, were visualized with high-resolution B-mode ultrasound. Preincubation in both specific and nonspecific microbubble suspension enhanced acoustic reflectivity of the thrombi. Abciximab immunobubbles targeted clots much more efficiently, showing a significant increase in echocontrast.

Results showing ultrasonic contrast enhancement in mean acoustic intensity are shown in Figure 3. Univariate analysis of variance demonstrates a highly significant difference ($P<0.0001$) in contrast enhancement for both groups. As shown in Figure 3, mean values of acoustic intensity of the clots were highest after preincubation in abciximab immunobubble suspension (37.84 $\pm$ 7.46 dB). The 6 clots exposed to nonspecific control microbubbles showed a mean value of 28.5 $\pm$ 4.14 dB compared with a mean value of 20.03 $\pm$ 2.84 dB for untreated native clots ($P=0.0014$). Differences in contrast enhancement were significant for abciximab immunobubble-targeted clots compared with both native control clots ($P=0.0006$, one-sided nonpaired Student $t$ test) and with clots with nonspecifically bound microbubbles ($P=0.014$).

**In Vitro Immunobubble Destruction and Reincubation**

The highly sensitive contrast agent imaging mode clearly depicted abciximab immunobubbles attached to the thrombus surface (Figure 4a). After bubble destruction for 30 seconds at a high MI, the clot was no longer visible (Figure 4b). After reincubation, the clot was again well visualized by ultrasound (Figure 4c).

**Molecular Imaging of Human Clots In Vivo**

For evaluation of the effectiveness of abciximab immunobubbles under in vivo conditions, human clots were inserted in the
right common carotid arteries of 19 male rats. Imaging with high-resolution B-mode ultrasound showed that not all clots were clearly detectable after insertion. The rate of detectable thrombi varied significantly among the groups ($P < 0.05$, $\chi^2$). Two of the 5 examined native control clots were poorly visualized and 3 were not detected. Six of the 7 thrombi preincubated in nonspecific immunobubbles were detectable showing either weak (50%) or moderate (50%) echogenicity. Of the 7 thrombi preincubated with abciximab immunobubbles, all were clearly visualized. One thrombus showed relatively weak echogenicity; all other thrombi showed high echocontrast in comparison to the vessel lumen (Figure 5a).

As shown in Figure 6, pretargeting the clots with abciximab immunobubbles resulted in a mean gray scale value of 50.09 ($\pm$ 3.73 SD); the mean gray scale value of the clots targeted with nonspecific immunobubbles was 32.69 ($\pm$ 6.91 SD) reflecting a highly significant contrast enhancement assessed by abciximab immunobubbles ($P < 0.0001$). Compared with the mean value of native control clots of 19.56 ($\pm$ 4.18 SD), both abciximab and Hu-Fab microbubble binding led to a significant increment of acoustic intensity ($P < 0.0001$ for abciximab immunobubbles; $P = 0.0037$ for Hu-Fab microbubbles), the latter resulting from nonspecific attachment of microbubbles to the thrombotic material.

Further experiments were performed to determine whether clot echogenicity could similarly be enhanced by intravenous application of targeted immunobubbles in our arterial occlusion model. Previously bound microbubbles, both specific and nonspecific, were first destroyed by ultrasound by increasing the MI from 0.05 to 1.3. Complete bubble destruc-
tion resulted in a remarkable loss of clot echogenicity both in the targeted and in the nontargeted microbubble group as depicted in Figure 5b. Targeted microbubbles were then delivered as a continuous infusion through a femoral vein catheter. This resulted in detectable clot enhancement in all 7 animals during administration of abciximab immunobubbles (see Figure 5c).

This enhancement was located primarily at the proximal portion of the thrombi (see arrow in Figure 5c). In three animals, distal portions of the clot were also enhanced by the abciximab immunobubbles. None of the thrombi were completely enhanced as compared with visualization after preincubation. Unlike the platelet-targeted immunobubbles, nonspecific immunobubble application failed to increase thrombus detectability after intravenous infusion.

Discussion

In this study, we showed that the glycoprotein IIb/IIIa receptor inhibitor abciximab can be attached to microbubbles for in vitro and in vivo molecular imaging of human clots. Our in vitro experiments demonstrated highly specific binding of abciximab immunobubbles to platelets with negligible binding of nonspecific immunobubbles. Using pulse inversion harmonic imaging, a contrast-specific imaging mode, we demonstrated that the echogenicity of human clots in vitro was significantly higher with specific abciximab immunobubbles as compared with nonspecific Hu-Fab immunobubbles, irrespective of immunobubble total encapsulated gas volume or MI. In our in vivo rat model of carotid occlusion with human thrombus, we likewise achieved highly specific imaging using abciximab immunobubbles. Thrombi preincubated with abciximab immunobubbles were clearly visualized as compared with control thrombi and with thrombi preincubated with nonspecific immunobubbles.

Complete vessel occlusion attributable to thrombosis leads to a considerable reduction in the amount of contrast agent that arrives at the clot surface for targeting and ultrasonographic visualization. A reduction of bound abciximab immunobubbles with reduced contrast enhancement could therefore result in inadequate in vivo imaging of thrombus in the clinical setting. Our experimental model therefore attempted to reflect thrombotic vessel occlusion through ligation of both external and internal carotid arteries distal to the location of the human thrombus that was inserted into the common carotid artery. Because human thrombi were well visualized in the rat carotid artery after incubation with abciximab immunobubbles, we were able to compare this “optimal” clot enhancement with that after intravenous application of targeted immunobubbles in a model of thrombotic occlusion. This was achieved by first destroying the incubation bubbles attached to the thrombus surface by means of transient high MI insonation and then infusing immunobubbles intravenously. Results demonstrated that the thrombus was still readily detectable with targeted bubbles. However, as compared with in vivo visualization of thrombi after incubation with abciximab immunobubbles, enhancement after intravenous application was located primarily to the proximal portion of the clot adjacent to the patent vessel lumen. Some enhancement in distal portions of the clot was also observed, but this was not a consistent finding. These results suggest that there was limited bubble diffusion into the thrombus. This may be the result of both low flow and immunobubble binding to receptors at the proximal clot surface. Clot enhancement was not achieved with systemic application of nonspecific immunobubbles. This finding is somewhat unexpected, because preincubation with nonspecific bubbles led to contrast enhancement in the in vitro experiments. This enhancement with nonspecific bubbles is most likely the result of inadequate washout of bubbles that are entrapped within the thrombus. Failure to enhance human thrombus with nonspecific immunobubbles in the in vivo occlusion model could also be attributed to nonspecific binding that may occur elsewhere in the animal, thus limiting the number of immunobubbles available for nonspecific binding to the thrombus. Moreover, the large differences in

Figure 4. Immunobubble destruction and reincubation. a, Human clot preincubated with abciximab immunobubbles. Arrows depict immunobubble attachment to clot surface. b, Clot after immunobubble destruction with a high MI (+). c, Molecular imaging of clot after second reincubation in abciximab immunobubbles. Clot now lies at a different position after immersion in a new saline bath as compared with a and b. Arrows show bubble attachment to clot surface.
enhancement (dB scale) between abciximab and nonspecific immunobubbles as demonstrated in the in vitro experiments will be applicable in the in vivo setting, thus limiting detection of smaller amounts of enhancement. Lastly, reduced flow to the thrombus resulting from arterial occlusion will also limit the amount of contrast agent available for molecular imaging.

This study implements in vivo ultrasonographic molecular imaging with a therapeutic drug. It is based on previous investigations in which specific imaging of therapeutically interesting targets was achieved through design of highly site-specific microbubbles that bind to vascular signatures such as integrins, selectin or activated platelets. In this study, we introduce novel immunobubbles binding abciximab for molecular imaging of human thrombus. Abciximab (ReoPro) is a licensed drug for prevention of platelet aggregation before percutaneous coronary intervention and after acute coronary syndrome. In a small open trial of abciximab for ischemic stroke within a 3- to 24-hour window, there was a significant improvement in National Institutes of Health Stroke Scale score and significant reduction in lesion size in the abciximab group. However, although first reports of an international randomized, double-blind, placebo-controlled phase 2 trial using abciximab (AbESTT Trial) within 6 hours of onset of ischemic stroke were encouraging, recent data from this study suggest an increased rate of intracerebral hemorrhage (unpublished data). To our knowledge, this is the first description of molecular imaging using a therapeutic drug attached to microbubbles. Takeuchi and coworkers developed microbubbles labeled with an arginine–glycine–aspartic acid peptide analog that binds to GPIIb/IIIa receptors on activated platelets. This compound has not been described for therapeutic use. In another study, an antifibrin monoclonal antibody (NIB 1H10) was used for visualizing porcine and canine thrombi. This substance also has no known therapeutic application. Eptifibatide (Integrilin) has been mixed with perfluorocarbon-exposed sonicated dextrose albumin for intracranial thrombolysis in pigs. Isotherm titration studies demonstrated an exothermic reaction between PESDA and eptifibatide, thus suggesting the existence of targeted bubbles. These bubbles were not used for molecular imaging.

Because abciximab is a humanized binding antigen fragment (Fab) of a monoclonal antibody against the glycoprotein IIb/IIIa receptor of platelets, it is an ideal candidate for attachment to microbubbles for the purpose of specific targeting of acute thrombosis. Besides allowing for detection of thrombus through targeted contrast agent enhancement, the specific binding of the microbubble to thrombus might allow for novel therapeutic applications. Insonation of clots has been shown to accelerate thrombolysis in several experimental settings. Likewise, recent clinical studies combining commercial 2 MHz transcranial Doppler devices with tissue plasminogen activator have shown high recanalization rates. There is also evidence that by combining ultrasound with microbubbles, thrombolysis may be performed even without...
recombinant tissue-type plasminogen activator. One study has demonstrated that clot lysis may be enhanced by sonication of a mixture of a platelet inhibitor and microbubbles. Because one mechanism for thrombolysis with combined ultrasound and microbubbles is related to microbubble cavitation, bringing the microbubble in close proximity to the thrombus through targeting with abciximab might enhance this effect. Because the concentration of abciximab bound to immunobubbles for such an application would be extremely small compared with the intravenous doses given in previous stroke trials, the risk of secondary hemorrhage would likely be significantly reduced. This interesting hypothesis will require careful studies to characterize the action and safety of such immunobubbles and compare their possible thrombolytic effects with nonspecific immunobubbles.

Study Limitations

Visualization of the carotid artery of the rat requires a very small, high-frequency transducer. This precludes using equipment with lower harmonic frequencies. Thus, a lack of contrast specific imaging may provide a partial explanation for the difficulty in imaging human thrombi in our carotid occlusion model after intravenous application of immunobubbles. In our in vitro experiments, we applied pulse inversion harmonic imaging that is customarily used for human diagnostics. This technique demonstrated the dramatic improvement of visualization of thrombi with immunobubbles. Such results are expected in the case of human imaging, which should actually be significantly better than in our vivo rat model of carotid occlusion. As can be anticipated, the in vivo model should be also applicable for testing clot lysis with abciximab immunobubbles. Despite limitations, our model of arterial occlusion should help us answer important questions regarding the feasibility of using abciximab immunobubbles for diagnosing, treating, and monitoring ischemic brain damage.

Sources of Funding

This project was funded by the European Union “Ultrasonographic Monitoring and Early Diagnosis of Stroke,” Contract No. QLG1-CT-2002-01518.

Disclosures

None.

References

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Stroke. 2007;38:1508-1514; originally published online March 22, 2007;
doi: 10.1161/STROKEAHA.106.471391
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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