Role of Plasminogen System Components in Focal Cerebral Ischemic Infarction

A Gene Targeting and Gene Transfer Study in Mice

N. Nagai, PhD; M. De Mol; H.R. Lijnen, PhD; P. Carmeliet, MD, PhD; D. Collen, MD, PhD

Background—The role of plasminogen system components in focal cerebral ischemic infarction (FCI) was studied in mice deficient in plasminogen (Plg<sup>-/-</sup>), in tissue or urokinase plasminogen activator (tPA<sup>-/-</sup> or uPA<sup>-/-</sup>), or in plasminogen activator inhibitor-1 or α<sub>2</sub>-antiplasmin (PAI-1<sup>-/-</sup> or α<sub>2</sub>-AP<sup>-/-</sup>).

Methods and Results—FCI was produced by ligation of the left middle cerebral artery and measured after 24 hours by planimetry of stained brain slices. In control (wild-type) mice, infarct size was 7.6±1.1 mm<sup>3</sup> (mean±SEM), uPA<sup>-/-</sup> mice had similar infarcts (7.8±1.0 mm<sup>3</sup>, P=NS), tPA<sup>-/-</sup> mice smaller (2.6±0.80 mm<sup>3</sup>, P<0.0001), PAI-1<sup>-/-</sup> mice larger (16±0.52 mm<sup>3</sup>, P<0.0001), and Plg<sup>-/-</sup> mice larger (12±1.2 mm<sup>3</sup>, P=0.037) infarcts. α<sub>2</sub>-AP<sup>-/-</sup> mice had smaller infarcts (2.2±1.1 mm<sup>3</sup>, P<0.0001 versus wild-type), which increased to 13±2.5 mm<sup>3</sup> (P<0.005 versus α<sub>2</sub>-AP<sup>-/-</sup>) after intravenous injection of human α<sub>2</sub>-AP. Injection into α<sub>2</sub>-AP<sup>-/-</sup> mice of Fab fragments of affinospecific rabbit IgG against human α<sub>2</sub>-AP, after injection of 200 μg human α<sub>2</sub>-AP, reduced FCI from 11±1.5 to 5.1±1.1 mm<sup>3</sup> (P=0.004).

Conclusions—Plg system components affect FCI at 2 different levels: (1) reduction of tPA activity (tPA gene inactivation) reduces whereas its augmentation (PAI-1 gene inactivation) increases infarct size, and (2) reduction of Plg activity (Plg gene inactivation or α<sub>2</sub>-AP injection) increases whereas its augmentation (α<sub>2</sub>-AP gene inactivation or α<sub>2</sub>-AP neutralization) reduces infarct size. Inhibition of α<sub>2</sub>-AP may constitute a potential avenue to treatment of FCI.

Key Words: plasminogen ■ plasminogen activators ■ cerebral infarction ■ cerebral ischemia

Neuronal degeneration in central nervous system diseases such as stroke, epilepsy, and Alzheimer’s disease is thought to be stimulated by an excess of the excitatory amino acid glutamate. Injection of glutamate agonists into the central nervous system indeed induces hippocampal neuronal cell death similar to that observed in neurodegenerative diseases. Excitotoxin-induced neuronal degeneration is mediated by tissue plasminogen activator (tPA). Consistent with this observation, mice deficient in tPA are resistant to, and infusion of plasminogen activator inhibitor-1 (PAI-1) protects against, excitotoxin-mediated hippocampal neuronal degeneration. Furthermore, deficiency of plasminogen (Plg), the zymogen substrate of tPA, and infusion of α<sub>2</sub>-antiplasmin (α<sub>2</sub>-AP), the physiological plasmin inhibitor, protect mice against excitotoxin-induced hippocampal neuronal death. It has been proposed that plasmin-mediated degradation of laminin sensitizes hippocampal neurons to cell death by disrupting neuron–extracellular matrix interaction.

Wang et al recently demonstrated that neuronal damage after focal cerebral ischemia induced by transient occlusion of the middle cerebral artery (MCA) was also reduced in mice with tPA deficiency and exacerbated by tPA infusion. Thus, the Plg system may be involved both in establishing a cerebral ischemic infarct and in its extension during thrombolytic therapy. We recently demonstrated that the neurotoxic effect of tPA on persistent focal cerebral ischemia also occurred with other thrombolytic agents, including streptokinase and staphylokinase. Thus, in patients with persistent cerebral arterial occlusion, thrombolytic therapy for ischemic stroke may cause infarct extension, which would not only partially offset the established overall beneficial effect of arterial recanalization but would indeed be harmful to a subgroup of patients. Because it is not possible to distinguish between patients who will and those who will not achieve cerebral arterial recanalization with thrombolytic therapy, the development of specific conjunctive strategies to counteract the neurotoxic effects of thrombolytic agents on persisting focal cerebral ischemia appears to be warranted.
the pathogenesis of cortical neuronal cell death has not been demonstrated. To delineate the contribution of individual components of the Plg (fibrinolytic) system on focal cerebral ischemic infarction, infarct size produced by ligation of the left MCA was quantified in mice with targeted inactivation of Plg, its activators tPA or uPA, or the fibrinolytic inhibitors PAI-1 or \( \alpha_2\)-AP. In addition, the effects of adenoviral transfer of the tPA and PAI-1 genes and of infusion of human \( \alpha_2\)-AP on cerebral infarction were studied.

Methods

Mice With Targeted Inactivation of Genes Encoding Plg System Components

All mice included in the present study were generated and bred at the Specific Pathogen Free Facility of the Center for Transgene Technology and Gene Therapy, Campus Gasthuisberg, KU Leuven, Belgium. Gene inactivation was obtained by homologous recombination in embryonic stem cells of the genes encoding tPA,\(^\text{12}\) urokinase plasminogen activator (uPA),\(^\text{12}\) PAI-1,\(^\text{13,14}\) Plg,\(^\text{15}\) or \( \alpha_2\)-AP,\(^\text{16}\) as previously described. Mice with inactivated genes encoding uPA receptor\(^\text{17}\) were not included because of the normal results obtained with uPA-deficient mice.

Adenovirus-Mediated Transfer of tPA or PAI-1 Genes

The recombinant adenoviruses AdCMVtPA and AdCMVPAI-1 were generated by homologous recombination in 293 cells essentially as previously described.\(^\text{18–20}\) After transfection, recombinant viral plaques were harvested and amplified as described.\(^\text{21–23}\) Large-scale production of recombinant adenovirus was performed as described.\(^\text{21}\) The kinetics and organ distribution of tPA and PAI-1 expression after adenoviral transfer by intravenous bolus injection have been described in detail elsewhere.\(^\text{24,25}\)

Injection of Human \( \alpha_2\)-AP and Affinospecific Rabbit Anti-Human \( \alpha_2\)-AP Fab Fragments

Human \( \alpha_2\)-AP was prepared from fresh-frozen plasma as previously described.\(^\text{26}\) Pooled rabbit antisera raised against human \( \alpha_2\)-AP were chromatographed on protein-A Sepharose, and affinospecific antibody bodies were obtained from the IgG pool by chromatography on a CNBr-activated Sepharose column substituted with human \( \alpha_2\)-AP, yielding \( \sim 0.1 \) mg specific IgG/mg applied. Fab fragments were obtained from the affinospecific IgG pool by digestion with 1% (wt/wt) papain in the presence of 50 mmol/L cysteine, 1 mmol/L EDTA, 0.1 mmol/L phosphate buffer, pH 7.0, for 5 hours. The reaction was arrested by addition of iodoacetamide to a final concentration of 75 mmol/L. After dialysis, the mixture was purified on a protein A Sepharose column equilibrated with PBS. Fab concentration was determined by ELISA calibrated against an IgG standard. SDS gel electrophoresis essentially revealed homogeneous Fab fragments (not shown).

Murine Cerebral Ischemic Infarction Model

Animal experiments were conducted according to the guiding principles of the American Physiological Society and the International Committee on Thrombosis and Hemostasis.\(^\text{27}\)

Focal cerebral ischemia was produced by persistent occlusion of the MCA according to Welsh et al.\(^\text{28}\) Briefly, mice of either sex, weighing 20 to 30 g, were anesthetized by intraperitoneal injection of ketamine (75 mg/mL, Aphaaero) and xylazine (5 mg/mL, Bayer). Atropine (1 mg/kg, Federa) was administered intramuscularly, and body temperature was maintained by keeping the animals on a heating pad. A U-shaped incision was made between the left ear and left eye. The top and back segments of the temporal muscle were transected, and the skull was exposed by retraction of the temporal muscle. A small opening (1 to 2 mm in diameter) was made in the region over the MCA with a handheld drill, with saline superfusion to prevent heat injury. The meninges were removed with a forceps, and the MCA was occluded by ligation with 10-0 nylon thread (Ethylon) and transected distally to the ligation point. Finally, the temporal muscle and skin were sutured back in place.

AdCMVtPA, AdCMVPAI-1, or AdRR5 was given as an intravenous bolus injection of \( 1.3 \times 10^9 \) cfu 4 days before ligation of the MCA. Human \( \alpha_2\)-AP (h\( \alpha_2\)-AP) was given intravenously, divided into 2 injections, given 1 minute before and 30 minutes after ligation of the MCA, respectively. Fab fragments were injected intravenously as a bolus 10 minutes after the second h\( \alpha_2\)-AP injection.

The animals were allowed to recover and were then returned to their cages. After 24 hours, the animals were killed with an overdose of Nembutal (500 mg/kg, Abbott Laboratories) and decapitated. The brain was removed and placed in a matrix for sectioning into 1-mm segments. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline,\(^\text{29}\) incubated for 30 minutes at 37°C, and placed in 4% formalin in PBS. With this procedure, the necrotic infarct area remains unstained (white) and is clearly distinguishable from stained (brick red) viable tissue (Figure 1). The sections were photographed and subjected to planimetry. The infarct volume was defined as the sum of the unstained areas of the sections multiplied by their thickness. A highly significant correlation has previously been observed between infarct size determined by TTC staining and clinical score \((r=0.67, P<0.0001)\) in rabbits 5 hours after thrombotic focal cerebral infarction.\(^\text{30}\)

Immunohistochemical Analyses

For immunostaining of laminin on paraffin sections, sections were incubated with a primary rabbit anti-laminin antibody (Sigma Chemical Co) diluted 1:50, followed by a peroxidase-labeled swine anti-rabbit IgG (Dako) diluted 1:50. Fibrin(ogen) was stained via a 3-step procedure with a goat anti-mouse fibrinogen (Nordic Immunologies) diluted 1:200, followed by rabbit anti-goat IgG (Dakopatts) diluted 1:100 and goat peroxidase-anti-peroxidase complex (Dako-patts) diluted 1:50. Peroxidase activity was developed by incubating sections in 0.05 mol/L Tris-HCl buffer (pH 7.0) containing 0.06% 3,3'-diaminobenzidine and 0.01% \( \text{H}_2\text{O}_2\), followed by counterstaining with hematoxylin.

Statistical Analysis

The data are represented as mean\( \pm \)SEM of n determinations. The significance of differences was determined by ANOVA followed by Fisher’s protected least significant difference test with the StatView software package.
Results

Cerebral Ischemic Infarct Size in Mice With Targeted Inactivation of Genes Encoding Plg System Components

Ligation of the left MCA induced a cerebral infarct with a volume of $7.6 \pm 1.1 \text{ mm}^3$ (n=11) in wild-type mice with a mixed (50% S129 and 50% C57BL/6) genetic background, a volume of $9.3 \pm 2.7 \text{ mm}^3$ (n=6) in inbred C57BL/6 mice, and a volume of $6.4 \pm 1.3 \text{ mm}^3$ (n=6) in inbred S129 mice ($P<0.05$ versus mixed background, results not shown).

Inactivation of the $tPA$ gene was associated with a significant reduction of infarct size to $2.6 \pm 0.8 \text{ mm}^3$ (n=11) ($P<0.0001$ versus wild-type mice), whereas inactivation of the $uPA$ gene had no effect on infarct size ($7.8 \pm 1.0 \text{ mm}^3$, n=8, $P=\text{NS}$ versus wild-type). Inactivation of the $PAI-1$ gene was associated with a significant increase in infarct size ($16 \pm 0.5 \text{ mm}^3$, n=6, $P<0.0001$ versus wild-type) (Figure 2). In mice with inactivated Plg genes, cerebral infarct size was significantly larger than in wild-type mice ($12 \pm 1.2 \text{ mm}^3$, n=9, $P=0.037$ versus wild-type), whereas, conversely, in $\alpha_2$-AP gene–deficient mice, infarct size was markedly reduced ($2.2 \pm 1.1 \text{ mm}^3$, n=7, $P=0.0001$ versus wild-type) (Figure 2).

Effect of $tPA$ and $PAI-1$ Gene Transfer on Cerebral Infarct Size

Injection of $1.3 \times 10^9$ pfu of AdCMVtPA in 6 $tPA^{-/-}$ mice 4 days before MCA ligation was associated with a cerebral infarct size of $6.0 \pm 1.3 \text{ mm}^3$, significantly larger than the infarcts in 5 $tPA^{+/+}$ mice injected with the control virus AdRR5 ($1.8 \pm 0.6 \text{ mm}^3$, $P=0.028$) (Figure 3A). Conversely, injection of $1.3 \times 10^9$ pfu of AdCMVPAI-1 in 5 $PAI-1^{-/-}$ mice was associated with a cerebral infarct size of $10 \pm 1.4 \text{ mm}^3$, significantly smaller than the infarcts in 5 $PAI-1^{+/+}$ mice injected with the control virus AdRR5 ($13 \pm 1.0 \text{ mm}^3$, $P=0.019$) (Figure 3B).

Effect of $\alpha_2$-AP on Cerebral Infarct Size

Cerebral infarct size correlated with $\alpha_2$-AP gene dose, corresponding to $11 \pm 2.0$, $4.9 \pm 2.0$, and $2.2 \pm 1.1 \text{ mm}^3$ in wild-type and heterozygously and homozygously deficient mice, respectively (Figure 4A). Injection of h$\alpha_2$-AP in $\alpha_2$-AP$^{-/-}$ mice increased the infarct size to $13 \pm 2.5 \text{ mm}^3$ (n=4) with a 1-mg total dose and to $11 \pm 1.5 \text{ mm}^3$ (n=6) with a 0.2-mg total dose. Injection of 1.7 mg affinospecific Fab against h$\alpha_2$-AP in mice given 0.2 mg h$\alpha_2$-AP reduced the cerebral infarct size to $5.1 \pm 1.1 \text{ mm}^3$ (n=7, $P=0.004$ versus 0.2 mg h$\alpha_2$-AP) (Figure 4B).

Figure 2. Effect of deficiency of Plg system components on focal ischemic cerebral infarct size. WT indicates wild-type (pooled values of 50% C57BL/6, 50% S129, 100% C57BL/6, and 100% S129 genetic background) and wild-type littermates of different gene-inactivated groups. Data represent mean values and SEM (vertical bars), with number of experiments given in columns.

Figure 3. Effect of adenoviral transfer of $tPA$ or $PAI-1$ genes on focal ischemic cerebral infarct size in $tPA^{-/-}$ or $PAI-1^{-/-}$ mice, respectively.

Figure 4. Effect of $\alpha_2$-AP on focal ischemic cerebral infarct size. A, Effect of $\alpha_2$-AP genotype on cerebral infarct size. B, Effect of injection of h$\alpha_2$-AP or of h$\alpha_2$-AP followed by anti–h$\alpha_2$-AP Fab fragments on cerebral infarct size.
In the present study, the effect of specific deficiencies of Plg system components on focal cerebral ischemic infarct size in mice was studied in an effort to gain further insight into the mechanism of infarct expansion by endogenous as well as exogenous tPA. A better understanding of this phenomenon might indeed lead to development of conjunctive treatments to reduce potential neurotoxic side effects of ischemic stroke and of thrombolytic therapy in patients with persisting focal cerebral ischemia.

The synergistic action of Plg system activation and excitotoxins on neuronal cell death in the hippocampus has been studied in significant detail. Using a mouse model with stereotactic injection of the glutamate analogue kainic acid in the hippocampus, they found that local tPA administration exacerbated whereas tPA deficiency or PAI-1 injection reduced hippocampal neuronal cell death. Furthermore, both Plg deficiency and local α2-AP injection protected against excitotoxin-induced neuronal degeneration. All findings were compatible with a mechanism in which tPA-mediated plasmin generation induced laminin degradation, resulting in disturbed neuron–extracellular matrix interaction. The extrapolation from these observations was that inhibitors of the hippocampal extracellular tPA/plasmin proteolytic cascade might protect neurons against excitotoxin-mediated brain disorders.

Focal cerebral ischemia induced by MCA occlusion is also associated with excitotoxin-mediated enhanced neuronal cell death and administration of tPA exacerbates persistent ischemic infarct size, suggesting that cortical ischemic neuronal cell death may be sensitive to a similar tPA/plasmin proteolytic cascade activation. This working hypothesis could not be confirmed in the present study, however. Whereas the findings to tPA deficiency protects against focal cerebral ischemic infarction were fully confirmed and extended by the observation that PAI-1 deficiency resulted in significantly larger infarcts, the observation that Plg deficiency protects against excitotoxin-induced neuronal cell death could not be confirmed. Instead, we found that focal cerebral infarct size was significantly larger in mice with Plg deficiency and, conversely, significantly smaller in mice with α2-AP deficiency. This internal consistency makes it unlikely that the discrepancy with the earlier observations on hippocampal neuronal degeneration might be coincidental or relate to differences in genetic background between the strains used in the two studies. Furthermore, although it has been shown that procedural and strain-related variables may significantly affect outcome in a murine model of focal cerebral ischemia, we have not observed significant differences in infarct size in mice with a C57BL/6, S129, or mixed background.

Immunohistochemical staining revealed the presence of laminin in the hippocampus but not outside the vessels in the ischemic or contralateral cortex. Fibrin(ogen) staining revealed increased extracellular reactivity in PAI-1−/− mice in the infarcted zone and its penumbra and some intravascular fibrin deposition in the infarcted region of the other genotypes, without a clear difference in density of immunoreactivity between Plg−/− and α2-AP−/− mice (apart from the differences in infarct size).

**Immunoneurochemical Staining**

Light-microscopic analysis of brain sections stained for laminin revealed clear immunoreactivity in the hippocampus but not outside the vessels in the ischemic or the viable cortex region (Figure 5A). Immunostaining of fibrin(ogen) revealed increased extracellular reactivity in PAI-1−/− mice (B.2) and comparable intravascular reactivity in other genotypes (tPA−/−, B.1; Plg−/−, B.3; and α2-AP−/−, B.4).

**Discussion**

In the present study, the effect of specific deficiencies of Plg system components on focal cerebral ischemic infarct size in mice was studied in an effort to gain further insight into the mechanism of infarct expansion by endogenous as well as exogenous tPA. A better understanding of this phenomenon might indeed lead to development of conjunctive treatments to reduce potential neurotoxic side effects of ischemic stroke and of thrombolytic therapy in patients with persisting focal cerebral ischemia.

The synergistic action of Plg system activation and excitotoxins on neuronal cell death in the hippocampus has been studied in significant detail. Using a mouse model with stereotactic injection of the glutamate analogue kainic acid in the hippocampus, they found that local tPA administration exacerbated whereas tPA deficiency or PAI-1 injection reduced hippocampal neuronal cell death. Furthermore, both Plg deficiency and local α2-AP injection protected against excitotoxin-induced neuronal degeneration. All findings were compatible with a mechanism in which tPA-mediated plasmin generation induced laminin degradation, resulting in disturbed neuron–extracellular matrix interaction. The extrapolation from these observations was that inhibitors of the hippocampal extracellular tPA/plasmin proteolytic cascade might protect neurons against excitotoxin-mediated brain disorders.

Focal cerebral ischemia induced by MCA occlusion is also associated with excitotoxin-mediated enhanced neuronal cell death and administration of tPA exacerbates persistent ischemic infarct size, suggesting that cortical ischemic neuronal cell death may be sensitive to a similar tPA/plasmin proteolytic cascade activation. This working hypothesis could not be confirmed in the present study, however. Whereas the findings to tPA deficiency protects against focal cerebral ischemic infarction were fully confirmed and extended by the observation that PAI-1 deficiency resulted in significantly larger infarcts, the observation that Plg deficiency protects against excitotoxin-induced neuronal cell death could not be confirmed. Instead, we found that focal cerebral infarct size was significantly larger in mice with Plg deficiency and, conversely, significantly smaller in mice with α2-AP deficiency. This internal consistency makes it unlikely that the discrepancy with the earlier observations on hippocampal neuronal degeneration might be coincidental or relate to differences in genetic background between the strains used in the two studies. Furthermore, although it has been shown that procedural and strain-related variables may significantly affect outcome in a murine model of focal cerebral ischemia, we have not observed significant differences in infarct size in mice with a C57BL/6, S129, or mixed background.

Immunohistochemical staining revealed the presence of laminin in the hippocampus but not outside the vessels in the ischemic or contralateral cortex. Fibrin(ogen) staining revealed increased extracellular reactivity in PAI-1−/− mice, suggestive of altered vascular permeability. Intravascular fibrin deposition was observed to a comparable extent in Plg−/− and α2-AP−/− mice, although the area in which this deposition occurred was much larger in the Plg−/− mice. In aggregate, our findings are incompatible with the unique linked cause-and-effect pathway demonstrated in the hippocampus (tPA-mediated plasmin generation, laminin degradation, and neuronal cell death). Instead, the data are suggestive of 2 independent mechanisms operating in opposite directions. At present, one can only speculate about possible mechanisms. tPA (and other exogenous thrombolytic agents) might affect the permeability of the blood-brain barrier, either directly or via activation of a substrate different from Plg, allowing excitotoxins to diffuse more readily and cause infarct extension. Altered permeability is suggested by the increased extracellular fibrin(ogen) deposition in the infarct zone of PAI-1−/− mice, but this will need further confirmation. Conversely, reduction of Plg activity might shift the hemostatic balance in the penumbra of the infarct toward transient intravascular deposition of fibrin. Although the density of fibrin deposition was not different between Plg−/− and α2-AP−/− mice, the total fibrin burden was much larger in the former because of the severalfold larger infarct size.

**Figure 5. Immunohistochemical analysis of laminin and fibrinogen.** A, Brain slice stained for laminin, which is abundant in hippocampus (A.1) but undetectable outside blood vessels in cortex (in both necrotic and viable areas, A.2). B, Brain slices stained for fibrinogen, revealing increased extravascular reactivity in PAI-1−/− mice (B.2) and comparable intravascular reactivity in other genotypes (tPA−/−, B.1; Plg−/−, B.3; and α2-AP−/−, B.4).
The internally consistent observations with α2-AP were unexpected but are potentially relevant for the treatment of ischemic stroke. First, a correlation was found between infarct size and genotype, with heterozygotes displaying infarct sizes between those of the wild-type and homozygous phenotypes. Second, bolus injection of hοα2-AP in α2-AP−/− mice caused a dose-related infarct expansion. Finally, and importantly, Fab fragments from affinosepecific polyclonal rabbit anti-hα2-AP antibodies significantly reduced the cerebral ischemic infarct size. This observation suggests that α2-AP inhibitors (eg, neutralizing monoclonal antibodies) might counteract focal ischemic infarction.

To be applicable to humans, the present observations obtained in a “heterologous and discontinuous” system, ie, using bolus hοα2-AP in α2-AP−/− mice, need to be extrapolated to the “autologous continuous” system in wild-type mice, eg, with a neutralizing monoclonal antibody. Efforts to produce monoclonal antibodies neutralizing murine α2-AP have been initiated by immunization of α2-AP−/− mice with purified murine α2-AP. The concentration of α2-AP in human plasma is 1 μmol/L, corresponding to a total body pool of ≈500 mg. An equivalent dose of a monoclonal Fab fragment would be ≈400 mg, which would be high but not excessive for a single administration. Furthermore, the observation that infarct size is proportional to the α2-AP level (derived from the gene dose effect and the dose-response effect of hοα2-AP transfusion) suggests that even a partial reduction of the plasma level might have a beneficial effect. In view of the excessive morbidity associated with ischemic stroke, further exploration of this potential avenue to reduction of focal cerebral ischemic infarct size would seem to be warranted.

References
Role of Plasminogen System Components in Focal Cerebral Ischemic Infarction: A Gene Targeting and Gene Transfer Study in Mice
N. Nagai, M. De Mol, H. R. Lijnen, P. Carmeliet and D. Collen

Circulation. 1999;99:2440-2444
doi: 10.1161/01.CIR.99.18.2440

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/18/2440

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/