Macrophage-Targeted Overexpression of Urokinase Causes Accelerated Atherosclerosis, Coronary Artery Occlusions, and Premature Death

Aaron E. Cozen, BA; Hideaki Moriwaki, MD, PhD; Michal Kremen, MD; Mary Beth DeYoung, PhD; Helén L. Dichek, MD; Katherine I. Slezicki; Stephen G. Young, MD; Murielle Véniant, PhD; David A. Dichek, MD

Background—Human atherosclerotic lesions contain elevated levels of urokinase plasminogen activator (uPA), expressed predominantly by macrophages.

Methods and Results—To test the hypothesis that macrophage-expressed uPA contributes to the progression and complications of atherosclerosis, we generated transgenic mice with macrophage-targeted overexpression of uPA. The uPA transgene was bred into the apolipoprotein E–null background, and transgenic mice and nontransgenic littermate controls were fed an atherogenic diet. uPA-transgenic mice had significantly elevated uPA activity in the atherosclerotic artery wall, of a magnitude similar to elevations reported in atherosclerotic human arteries. Compared with littermate controls, uPA-transgenic mice had accelerated atherosclerosis, dilated aortic roots, occlusive proximal coronary artery disease, myocardial infarcts, and early mortality.

Conclusions—These data support the hypothesis that overexpression of uPA by artery wall macrophages is atherogenic and suggest that uPA inhibitors might be therapeutically useful. (Circulation. 2004;109:2129-2135.)

Key Words: urokinase ■ atherosclerosis ■ coronary disease

Urokinase plasminogen activator (uPA) is expressed by human arterial wall macrophages1–3 and may contribute to the pathogenesis of atherosclerosis. uPA expression is elevated in atherosclerotic human arteries, ≈2- to 5-fold above levels in normal arteries.1–4 Moreover, within individual coronary arteries, the level of local uPA expression is directly correlated with atherosclerosis severity.3 Elevated uPA expression may also be associated with complications of atherosclerosis including aneurysm formation and plaque rupture. uPA expression is increased in human aneurysms,5 and uPA acts via plasmin to activate collagenases,6 which are potential triggers of plaque rupture.7 However, few prospective experimental data support a causal link between uPA and atherosclerosis.

Studies in genetically modified mice8–13 do not, in general, support an atherogenic role for uPA. For example, apolipoprotein E–null (Apoe–/–) mice also deficient in uPA had the same amount of atherosclerosis as uPA-expressing controls.8 In 3 separate studies, Apoe–/– mice deficient in plasminogen activator inhibitor 1 (PAI-1; the primary physiological inhibitor of uPA) had either the same,10 less,11 or more atherosclerosis12 than wild-type mice. Apoe–/– mice deficient in plasminogen (the primary physiological substrate of uPA) had increased atherosclerosis,9 consistent with antiatherogenic roles for plasminogen and its activator, uPA. Apoe–/– mice with macrophage-specific overexpression of collagenase had decreased atherosclerosis.13 This study is relevant to uPA because collagenases are activated by the uPA/plasminogen system; therefore, the effect of elevated collagenase activity might predict the effect of elevated uPA. Decreased atherosclerosis in collagenase-overexpressing mice together with increased atherosclerosis in plasminogen-null mice supports a general hypothesis that elevated arterial wall proteolytic activity decreases atherosclerosis.14 This hypothesis predicts that elevated arterial uPA expression would be antiatherogenic.

Although many of these murine atherosclerosis studies predict an antiatherogenic role for uPA, virtually all of these studies involve manipulation of molecules other than uPA. In the single study in which uPA expression was manipulated directly, uPA expression was eliminated entirely.8 In contrast, uPA expression is increased in atherosclerotic human arteries.1–3 We hypothesized that uPA has atherogenic activity that is manifested when it is expressed at elevated levels by...
macrophages. This hypothesis was supported by experiments in which gene transfer of uPA to the carotid arteries of hypercholesterolemic rabbits increased intimal growth. However, in this short-term study, uPA was expressed in endothelial cells, whereas macrophages are the primary cell type that expresses uPA in human lesions. To test the hypothesis that macrophage-expressed uPA is atherogenic, we generated transgenic mice with macrophage-targeted overexpression of uPA, bred the transgene into the Apoe−/− background, and evaluated the development of atherosclerosis.

Methods

Generation of Transgenic Mice
A macrophage-targeted uPA transgene was constructed by fusing promoter and enhancer sequences from the human scavenger receptor A gene (from C. Glass, University of California, San Diego) to the mouse uPA gene (from J. Degen, Children’s Hospital Medical Center, Cincinnati, Ohio). This ‘SR-uPA’ transgene was used to generate transgenic mice in the C57BL/6 × SJL background. All animal protocols were approved by the institutional animal care and use committees.

Atherosclerosis Studies
The SR-uPA transgene was established in the C57BL/6 Apoe−/− background by serial breedings with C57BL/6 Apoe−/− mice (Jackson Laboratories, Bar Harbor, Maine). SR-uPA Apoe−/− mice were identified by Southern analysis of tail-tip DNA and Western blotting of plasma to confirm the absence of apo E. Mice in the atherosclerosis studies were at least 94% C57BL/6 background. All atherosclerosis studies compare transgenic mice and nontransgenic littermates, which controls for the limited numbers (≤6%) of non-C57BL/6 genes. Female mice 5 weeks of age were begun on a diet containing 21% fat and 0.15% cholesterol by weight (TD88137; Harlan-Teklad).

Plasma Lipids
Cholesterol and triglyceride levels were measured (Spectrum cholesterol assay, Abbott, and TG triglyceride kit, Boehringer Mannheim). Lipoproteins were fractionated by fast protein liquid chromatography (FPLC).

Northern Analysis
Organs were snap-frozen and stored at −80°C, and RNA was extracted with TRIzol (Gibco BRL). Macrophage RNA was obtained by applying TRIzol directly to the cultured cells. Blots were hybridized to uPA and GAPDH cDNA probes. Bound probes were quantified with a phosphorimager.

Macrophage Plasminogen Activation
Macrophages were collected by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycollate solution (Difco). Peritoneal cells were plated, nonadherent cells were removed, and medium was replaced with M199 (without phenol red), including the plasmin substrate S-2390 (Chromogenix) and human Glu-plasminogen (American Diagnostica). Plasminogen activation was measured as the change in absorbance at 405 nm and the level of uPA expression calculated as ΔOD405 nm.

Aortic uPA Activity
After 5 or 10 weeks on the atherogenic diet, anesthetized mice were perfused with saline, and their thoracic aortas were snap-frozen and stored at −80°C. Aortas were pulverized and homogenized in 400 μL of buffer consisting of (in mmol/L) 75 acetic acid, pH 4.2, 75 KCl, 225 NaCl, 10 EDTA, and 100 arginine, and 0.25% Triton X-100, and centrifuged at 12 000g. The supernatant was frozen at −80°C. Protein concentration in the extracts was determined with the BCA assay (Pierce), and uPA activity was measured in aliquots diluted to 0.8 μg/mL protein. To convert single-chain uPA to the active 2-chain uPA, 20 μL of extract was added to 100 μL of buffer including 50 mmol/L Tris, pH 8.8, 38 mmol/L NaCl, 0.1% BSA, and 10 μg/mL human plasmin (American Diagnostica). After 30 minutes at 37°C, 2 μL of aprotinin (1.2 trypsin inhibitor units/mL, Sigma) was added to inhibit plasmin and other proteases. The uPA substrate S-2444 (Chromogenix) was added at 0.48 mg/mL. Absorbance at 405 nm was measured immediately and after 17 hours. Conversion of single-chain uPA to 2-chain uPA by plasmin treatment and addition of aprotinin maximizes specificity for uPA.

The reliability of conversion of single-chain to 2-chain uPA and the linearity and sensitivity of the uPA assay were confirmed by assay of human single-chain uPA (American Diagnostica) in parallel.

Tissue Processing and Histology
Pinned aortas were stained with Sudan IV. For frozen sectioning, hearts and aortic roots were processed into OCT compound (VWR). Other hearts were placed overnight in fixative, then processed into paraffin.

Eighty 10-μm-thick serial cryosections were cut from each aortic root, beginning at the level of attachment of the aortic valve cusps. Serial sections were stained with hematoxylin and eosin, oil red O, Movat’s pentachrome, and the macrophage-specific antibody MOMA-2 (Biosource). Sections of paraffin-embedded hearts were stained with hematoxylin and eosin or Masson’s trichrome.

Aortic root sections from 4 transgenic and 5 nontransgenic mice (all Apoe−/−) were stained with an antibody to smooth muscle α-actin (Clone 1A4, Dako) or control mouse IgG directed against an irrelevant epitope (Dako U 0951).

Morphometry
Total intimal area, intimal oil red O–stained and MOMA-2–stained areas, and lumen circumference were measured on 4 to 8 evenly spaced step sections per aortic root by use of computer-assisted color thresholding and planimetry. Total luminal surface and stained areas of Sudan IV–treated, pinned aortas were quantified.

Quantification of Coronary Artery Stenosis
Two observers, blinded to genotype, evaluated all oil red O–stained aortic root sections to identify the major epicardial coronary arteries and locate, for each artery, the section with the maximal stenosis. Images of these sections were used to measure, along a diameter extending across the maximal stenosis, the original lumen diameter (ie, distance between the inner edges of the media) and the residual lumen diameter. Percent stenosis=(original lumen diameter−residual lumen diameter/original lumen diameter)×100%.

Peripheral Blood Cell Counts
Counts were performed by an outside laboratory (Phoenix Central Laboratory).

Statistical Methods
Group means were compared by unpaired t test, except for percentage stenosis, for which the rank-sum test was used. Accuracy of genotyping based on assessment of medial destruction was evaluated by χ2 testing. Survival probabilities were compared by the log-rank test.

Results
Expression of uPA in Founder Lines
Of 28 pups, 5 transmitted the transgene to their offspring. To determine the level and extent of macrophage-specific uPA expression, freshly harvested organs and thioglycollate-stimulated, cultured peritoneal macrophages were studied by Northern analysis. Macrophages were also assayed for plas-
phages. SR-uPA transgene is expressed predominantly in macrophages. Data are representative of 2 independent experiments, performed with RNA from different mice (4 total). A third experiment, performed with 8 additional mice, further quantified uPA mRNA levels in macrophages (see Results). B, Plasminogen activator activity. Thioglycollate-stimulated peritoneal macrophages of transgenic (SR-uPA; n=3) and nontransgenic littermate control mice (n=2; essentially identical results were obtained with 5 other nontransgenic mice). Ex vivo macrophages were incubated with plasminogen and plasmin substrate S-2390, and plasmin activity was measured as OD405 of H11005 and nontransgenic control mice (n=2; essentially identical results were obtained with 5 other nontransgenic mice). Ex vivo macropodocytes were harvested and placed in culture before RNA extraction. Top, Blots were probed with a uPA cDNA. Endogenous uPA transcript is 2.9 kb; SR-uPA (transgene) transcript is 1.9 kb. Bottom, Ethidium bromide-stained gels. Endogenous uPA is expressed primarily in kidney with low levels in thioglycollate-stimulated peritoneal macrophages. Data are mean±SD.

Comparison of Transgenic (SR-uPA+/-) and Nontransgenic (SR-uPA0/0) Mice

<table>
<thead>
<tr>
<th></th>
<th>SR-uPA+/-</th>
<th>SR-uPA0/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood monocytes, per µL</td>
<td>160±117 (n=5)</td>
<td>195±259 (n=5)</td>
</tr>
<tr>
<td>Peripheral blood monocytes, % total leukocytes</td>
<td>1.6±1.5 (n=5)</td>
<td>1.8±2.2 (n=5)</td>
</tr>
<tr>
<td>Aortic uPA activity, IU/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk diet</td>
<td>0.20±0.047* (n=5)</td>
<td>0.136±0.038 (n=6)</td>
</tr>
<tr>
<td>10-wk diet</td>
<td>0.35±0.087† (n=8)</td>
<td>0.16±0.037 (n=8)</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk diet</td>
<td>1193±344 (n=6)</td>
<td>1496±104 (n=6)</td>
</tr>
<tr>
<td>10-wk diet</td>
<td>1073±279 (n=8)</td>
<td>1309±179 (n=9)</td>
</tr>
<tr>
<td>Aortic root intimal area, µm²×10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk diet</td>
<td>9.5±3.2† (n=16)</td>
<td>4.3±1.3 (n=11)</td>
</tr>
<tr>
<td>10-wk diet</td>
<td>6.2±2.1† (n=16)</td>
<td>2.5±0.51 (n=11)</td>
</tr>
<tr>
<td>Aortic root oil red 0 area, µm²×10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-wk diet</td>
<td>65.9±7.9 (n=16)</td>
<td>61.1±15 (n=11)</td>
</tr>
<tr>
<td>10-wk diet</td>
<td>62.5±6.8 (n=16)</td>
<td>61.4±4.4 (n=9)</td>
</tr>
<tr>
<td>Sudanophilic lesions, % of total aortic lumen surface area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic root lumen circumference, mm</td>
<td>5.7±0.68* (n=16)</td>
<td>5.2±0.28 (n=11)</td>
</tr>
<tr>
<td>Maximal coronary stenosis, % of lumen narrowing</td>
<td>62‡ (40–83) (n=23)</td>
<td>26 (0–17) (n=17)</td>
</tr>
</tbody>
</table>

Values are mean±SD except for coronary stenoses, which are median and (25%–75%) range. n indicates mice in each group except for coronary stenosis, for which n indicates arteries. Variations in n between groups are because of omission of specimens of suboptimal technical quality.

P values are for difference from nontransgenic controls: *P≤0.05; †P≤0.01; ‡P≤0.001.
Lipid Profiles

Transgenic mice could be compared.

Reference range with which aortic uPA activity in the rotic human arteries is elevated 2- to 5-fold, there is a aortas. Because uPA expression and activity in atherosclerotic and measured uPA activity in extracts of atherosclerotic sections and sudanophilic area on pinned aortas were significantly increased in transgenic mice (2-fold; Table; Figure 2, A and B).

Macrophage gene expression in vivo. For these reasons, we bred the transgene into the C57BL/6 Apoe Apo e-/- background and measured uPA activity in extracts of atherosclerotic aortas. Because uPA expression and activity in atherosclerotic human arteries is elevated 2- to 5-fold, there is a reference range with which aortic uPA activity in the transgenic mice could be compared.

uPA Activity Is Significantly Elevated in Aortas of SR-uPA Apo e-/- Mice

uPA activity was significantly elevated in aortas of transgenic SR-uPA Apo e-/- mice (Table). The magnitude of elevated uPA activity in 10-week transgenic aortas (2.2-fold) was within the range reported in atherosclerotic human arteries. There is a reference range with which aortic uPA activity in the transgenic mice could be compared.

SR-uPA Apo e-/- Mice Have Unaltered Plasma Lipid Profiles

SR-uPA Apo e-/- mice had no significant alterations in plasma cholesterol, triglycerides, or FPLC profiles (Table; online Data Supplement).

Peripheral Blood Monocyte Counts

Neither total peripheral monocyte counts nor percentage of peripheral blood monocytes differed between transgenic and nontransgenic mice (Table; P>=0.8 for both comparisons).

Increased Atherosclerosis in SR-uPA Apo e-/- Mice

After 10 weeks on diet, both intimal area in aortic root sections and sudanophilic area on pinned aortas were significantly increased in transgenic mice (2-fold; Table; Figure 2, A and B).

Plaque Structure and Composition Are Unaltered in SR-uPA Apo e-/- Mice

Total plaque macrophage and lipid areas in aortic root cross sections were both significantly increased in transgenic mice (2-fold; Table). However, transgenic plaques were also proportionately larger; therefore, the percentage of plaque occupied by macrophages and lipids did not differ between transgenic and nontransgenic mice (Table; P=0.3 for both). Smooth muscle alpha-actin staining was essentially limited to a thin layer along the surface of some of the plaques and did not differ between transgenic and nontransgenic mice. No differences in collagen, proteoglycan, or elastin staining were discernible in Movat-stained sections of transgenic and nontransgenic intimal plaques.

SR-uPA Apo e-/- Mice Have Dilated Aortic Roots and Increased Medial Destruction

Aortic root lumen circumferences were significantly larger in transgenic mice. This dilation was only focal, however, because the total luminal surface area of transgenic aortas was not increased (Figure 2, A and B, and the Table). The appearance of the media in individual aortic root sections ranged from essentially intact to severe focal destruction (Figure 2, C and D). Medial destruction (loss of both matrix and smooth muscle cells) appeared more severe in transgenic mice. To evaluate this impression objectively, an observer blinded to genotype was given a single Movat-stained aortic root section from each of 7 transgenic and 7 nontransgenic mice and asked to assign a genotype based only on the extent of medial destruction. Twelve of 14 mice (86%) were correctly genotyped (P<0.01).

SR-uPA Apo e-/- Mice Have Severe Occlusive Proximal Coronary Artery Disease, Extensive Myocardial Infarcts, and Early Mortality

We enrolled mice in a longer-term study to determine whether they developed aneurysms or plaque rupture. However, transgenic mice began to die suddenly beginning at 15 weeks of age (Figure 3). Mice that died suddenly or were killed at 15 weeks of age (for the atherosclerosis study) had gross and histological evidence of myocardial infarcts (Figure 4, A and C) as well as significant stenoses and total occlusions of proximal coronary arteries (Figure 4, D through
null mice might instead result from secondary, systemic alternatives, accelerated atherosclerosis in plasminogen absence of plasmin or by an excess of plasmin activity. This suggests that atherosclerosis may be accelerated either by an increase in uPA mice (which have increased arterial wall plasminogen had increased atherosclerosis. In contrast, the SR- macrophages. First, mice deficient in uPA had unaltered atherosclerosis but did not alter plaque structure; (3) uPA-overexpressing mice died suddenly and prematurely, with severe proximal coronary atherosclerosis and myocardial infarcts. Therefore, macrophage-expressed uPA contributes to the progression and complications of atherosclerosis.

Our results must be reconciled with atherosclerosis studies performed with mice deficient in uPA, plasminogen, or PAI-1 and with mice overexpressing collagenase in macrophages. First, mice deficient in uPA had unaltered atherosclerosis.8 In contrast, uPA overexpression in macrophages increases atherosclerosis. uPA may accelerate atherosclerosis only when it is expressed above a critical level. Other factors that may explain why uPA deficiency did not decrease atherosclerosis include use of a more atherogenic diet (1.25% cholesterol with added cholic acid) and the apparent use of mice with mixed genetic backgrounds.8 Second, Apoe<sup>−/−</sup> mice deficient in plasminogen had increased atherosclerosis.9 In contrast, the SR-uPA mice (which have increased arterial wall plasminogen activation) have increased atherosclerosis. This result suggests that atherosclerosis may be accelerated either by an absence of plasmin or by an excess of plasmin activity. Alternatively, accelerated atherosclerosis in plasminogen-null mice might instead result from secondary, systemic effects of the wasting disease that these mice develop.28 Third, our results are consistent with a recent study showing increased atherosclerosis in PAI-1 null mice.12 Finally, mice overexpressing collagenase in macrophages had elevated artery wall proteolytic activity but less atherosclerosis.13 In contrast, elevated artery wall proteolytic activity in SR-uPA mice increased lesion size. This discrepancy is informative because it confirms the specificity of our results. Accelerated atherosclerosis in SR-uPA mice is not a nonspecific effect of protease overexpression in macrophages; rather, it is a specific effect of uPA overexpression.

The inconsistency between the substantial peritoneal macrophage uPA overexpression detected ex vivo (Figure 1) and the modest uPA overexpression measured in aortic extracts (Table) is striking. Another line of SR-uPA transgenic mice also showed a large discrepancy between peritoneal macrophage uPA expression (estimated 10-fold increase) and uPA expression in aortic tissue (no increase). Although it is probable that the absolute level of uPA activity (IU/mg protein) is higher within the macrophage-rich aortic lesions than in whole aortic extracts, it is also likely that the ex vivo assay, performed in nonphysiological conditions using peritoneal macrophages, overestimates uPA overexpression in vivo in aortic wall macrophages. Alternatively, and far less likely, a tremendous increase in uPA-mediated proteolytic activity in vivo increases atherosclerosis only modestly and is insufficient to alter plaque structure.

There are several mechanisms through which uPA could accelerate atherosclerosis. uPA might increase macrophage migration into the intima.29 uPA could increase cell proliferation by activating or releasing matrix-bound growth factors.30,31 uPA might also bind to receptors on vascular cells, stimulating proliferation and migration.32 uPA-stimulated proteolysis could increase lipid accumulation by cleaving artery wall matrix proteins, creating binding sites for atherogenic lipoproteins.33 It is likely that uPA is acting locally, within the plaque, to accelerate atherosclerosis, because among the 3 lines of mice that we studied, only the line with elevated artery wall uPA expression had increased atherosclerosis. Systemic effects of uPA are less likely, because there was no effect of uPA.
overexpression on plasma lipids (Table; online Data Supplement), and we did not encounter neonatal bleeding or hepatocellular carcinoma, which occur in mice with generalized uPA overexpression.\textsuperscript{34,35}

To identify a cause for the sudden deaths of the transgenic Apoe\textsuperscript{-/-} mice, we examined several organs postmortem. Only the hearts and aortas appeared abnormal, and we did not observe either peripheral edema or aortic rupture. Thus, the deaths were not caused by chronic heart failure or aneurysmal disease. The presence of myocardial infarcts along with occlusive coronary disease suggests that the deaths were caused by cardiac arrhythmias. Further studies may reveal whether the SR-uPA Apoe\textsuperscript{-/-} mice are a useful model of sudden death caused by atherosclerotic cardiovascular disease.

The combination of elevated macrophage proteolytic activity, sudden death, and coronary occlusions suggested that macrophage-expressed uPA might cause plaque rupture. However, the occlusions appeared to result from aortic plaque extension into coronary ostia (Figure 4, D through F). Plaque rupture may not have occurred because the lesions were at an early stage, without necrotic cores. Mice with macrophage-specific expression of collagenase also did not manifest plaque rupture.\textsuperscript{13} It remains a challenge to provide experimental support for the hypothesis that elevated lesion proteolytic activity causes plaque rupture.

The finding of dilated aortic roots in SR-uPA mice supports a role for artery wall uPA activity in aneurysm formation. The absence of frank aortic aneurysms is most likely a result of the early deaths of SR-uPA mice. The aortic root dilation contrasts with a previous study in which brief overexpression of uPA in endothelial cells of rabbit carotid arteries caused arterial constriction.\textsuperscript{15} There are 2 likely reasons for this. First, the vascular media in the present study may have been sufficiently damaged by uPA overexpression (Figure 2D) that it could no longer sustain contractile activity. Alternatively, constriction could be caused by an endothelial cell–specific activity of uPA, such as cleavage of a vasoactive peptide.\textsuperscript{36}

In summary, our data support the hypothesis that elevated uPA expression in arterial wall macrophages contributes to occlusive vascular disease and to aneurysm formation. SR-uPA mice may provide a useful model of sudden death associated with coronary atherosclerosis. Elevated arterial wall uPA activity in humans may be a target for therapies that slow atherosclerosis.

Acknowledgments

This work was supported by grants from the University of California Tobacco-Related Disease Research Program (7RT-0016), the National Institutes of Health (NIH) (HL-61860 and HL-69063), and the Howard Hughes Medical Institute Research Resources Program (to Dr D. Dichek). Dr DeYoung and Dr H. Dichek were supported by awards from the NIH (F32-HL-10022 and K08-HL-04031). The authors thank Brian Kelley and Ruth Linnemann for assistance with experiments and Gary Howard, Stephen Ordway, and Margo Weiss for editorial advice and assistance.

References


Macrophage-Targeted Overexpression of Urokinase Causes Accelerated Atherosclerosis, Coronary Artery Occlusions, and Premature Death
Aaron E. Cozen, Hideaki Moriwaki, Michal Kremen, Mary Beth DeYoung, Helén L. Dichek, Katherine I. Slezicki, Stephen G. Young, Murielle Véniant and David A. Dichek

_Circulation_. 2004;109:2129-2135; originally published online April 19, 2004;
doi: 10.1161/01.CIR.0000127369.24127.03
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/109/17/2129

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2004/05/03/01.CIR.0000127369.24127.03.DC1

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/