Influence of C3 Deficiency on Atherosclerosis

Chiara Buono, MD*; Carolyn E. Come, BA*; Joseph L. Witztum, MD; Graham F. Maguire, BSc; Philip W. Connelly, PhD; Michael Carroll, PhD; Andrew H. Lichtman, MD, PhD

Background—The influence of complement activation on atherosclerosis is not well understood. The purpose of this study was to examine the effects of C3 deficiency on the extent and phenotype of atherosclerosis.

Methods and Results—Aortic atherosclerosis was analyzed in low-density lipoprotein receptor (ldlr)/C3-deficient mice (ldlr<sup>−/−</sup>/C3<sup>−/−</sup>) and ldlr<sup>−/−</sup>/C3<sup>+/+</sup> littermate control mice after 15 weeks on a 1.25% (wt/wt) cholesterol diet. Serum lipoprotein profiles and immunoglobulin levels were not significantly different between the 2 experimental groups. The lipid-positive en face lesion area in thoracic and abdominal aorta was greater in C3-deficient mice than in control mice (3.9% versus 2.1%, median, \( P=0.0076 \)). Similarly, the lipid-positive area in aortic arch sections was greater in C3-deficient mice than in controls (0.04 mm<sup>2</sup> versus 0.02 mm<sup>2</sup>, median, \( P=0.0089 \)). Analysis of aortic arch sections showed greater lesional macrophage content in C3-deficient versus control mice (8.24±1.36% versus 5.9±1.63% intimal area, mean±SEM, \( P=0.003 \)), less smooth muscle cell content in C3-deficient versus control mice (0.06±0.05% versus 0.92±0.32% intimal area, mean±SEM, \( P<0.0001 \)), and less collagen content in C3-deficient versus control mice (0.52±1.26% versus 11±10.43% intimal area, mean±SEM, \( P=0.008 \)).

Conclusions—The maturation of atherosclerotic lesions beyond the foam cell stage is strongly dependent on an intact complement system. (Circulation. 2002;105:3025-3031.)

Key Words: atherosclerosis • complement • inflammation

atheromas are chronic inflammatory lesions of the vessel wall in which many components of the innate and adaptive immune system are active.1 Soluble immune effector molecules frequently found in arterial lesions include antibodies and complement proteins. The complement system plays a central role in protective immune responses to microbial pathogens and in the pathogenesis of immune-mediated disease processes. Complement activation, either by the antibody-dependent classical pathway or the alternative or lectin pathways, generates proinflammatory mediators such as C3a and C5a, which can activate endothelium and enhance leukocyte recruitment to inflammatory sites. C3 fragments bind covalently to antibodies or cell surfaces, thereby acting as opsonins that could enhance phagocytosis by macrophages. Generation of the terminal membrane attack complex (MAC) on the surface of lesional cells may kill these cells. Furthermore, complement is involved in the stimulation and regulation of antibody responses. In particular, the CD19/CD21 receptor complex on B cells links the complement system to the activation of B cells.2 In addition, the CD21 complement receptor expressed on follicular dendritic cells contributes to the maintenance of memory B cells and B-cell tolerance to self-antigens.3 Mice deficient in C3 have diminished protective inflammatory responses to infections, a profound defect in their antibody response to T-dependent antigens, diminished germinal center formation, and a failure in isotype switching.4,5 Individuals with atherosclerotic disease produce antibodies specific to atheroma antigens, such as oxidized low-density lipoproteins (LDLs)6 and heat shock protein 60/65.7 Antibody responses to oxidation-specific epitopes of oxidized LDL may modulate atherosclerotic disease.8,9 However, the mechanisms by which these antibodies influence atherogenesis are not understood. Because humoral immune responses to certain antigens are associated with and may modulate atherogenesis and because the complement system is involved in both the inductive and effector phases of humoral immune responses, it is plausible to hypothesize that complement is involved in modulating atherogenesis. Furthermore, antibody-independent activation of complement in arterial lesions may contribute to the inflammatory process of atherosclerosis.

Several studies provide evidence that complement activation is involved in atherogenesis. Components of the terminal complement pathway are frequently found in human atheromas.10–12 C3 and C4 deposition in arterial lesions has also

Received February 25, 2002; revision received April 3, 2002; accepted April 8, 2002.
From the Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School (C.B., C.C., A.H.L.) and Center for Blood Research and Department of Pediatrics, Harvard Medical School (M.C.), Boston, Mass; Department of Medicine (J.L.W.), University of California, San Diego, La Jolla, Calif; and J. Alick Little Lipid Research Laboratory (G.M., P.W.C.), St Michael’s Hospital, Toronto, Ontario, Canada.
*These authors contributed equally to this study.

Correspondence to Dr Andrew H. Lichtman, Department of Pathology, Brigham and Women’s Hospital, 221 Longwood Ave, Boston, MA 02115.
E-mail alichtman@rics.bwh.harvard.edu
© 2002 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000019584.04929.83

3025
been demonstrated.\textsuperscript{13,14} Furthermore, RNA analysis indicates that complement genes are expressed locally within the plaques.\textsuperscript{15} Although there is evidence for complement activation in atherosclerotic lesions, little is known about how complement is activated in lesions or the influence of complement activation on disease progression or phenotype. Several components of the arterial wall may trigger complement activation and C5b-9 assembly. The classical pathway of complement may be activated by C-reactive protein (CRP) bound to enzymatically degraded LDL.\textsuperscript{16} Consistent with this view is the finding that the terminal complement proteins colocalize with CRP in the intima of early human lesions.\textsuperscript{17} Likewise, immunoglobulin (Ig) M or IgG antibodies specific for oxidized LDL within lesions may activate the classical pathway.\textsuperscript{18} \textit{Chlamydia pneumoniae}, a microbe frequently associated with atherosclerotic lesions, may also activate the alternative pathway of complement.

A limited number of studies on the influence of complement protein deficiencies on atherogenesis have been reported. In rabbits, there is an apparent protective effect of C6 complement protein deficiencies on atherogenesis.\textsuperscript{19} In contrast, a C8-deficient strain of rabbits has been demonstrated to accelerate atherogenesis.\textsuperscript{20} Therefore, the knowledge of the influence of complement genes on atherogenesis is incompletely elucidated. Several components of the arterial wall may trigger complement activation on disease progression or phenotype. Therefore, we compared extent and phenotype of diet-induced atherosclerotic lesions in LDLR-deficient (ldlr$^{-/-}$) mice with or without C3 deficiency.

**Methods**

**Mice**

Male ldlr$^{-/-}$ mice, backcrossed on the C57Bl/6J background 6 times, purchased from Jackson Laboratories (Bar Harbor, Maine), were crosbred with female C3$^{-/-}$ mice* on a mixed 129/B6 background to generate ldlr$^{-/-}$ C3$^{-/-}$ double-knockout mice and ldlr$^{-/-}$ C3$^{+/+}$ littermate controls. The C3 genotype was identified by polymerase chain reaction with the use of LDLR gene–specific primers,\textsuperscript{21} whereas the C3 genotype was followed by ELISA determination of serum C3 protein levels (sensitive to $\approx 5$ ng/mL), as described.\textsuperscript{22} C3$^{-/-}$ heterozygous littersates were used as controls to ensure that enough age-matched, genetically comparable mice were available for the studies. The serum C3 levels in C3$^{-/-}$ heterozygotes were not significantly different from wild-type C3$^{+/+}$ mice, and the C3$^{-/-}$ heterozygotes do not have any detectable phenotypic differences from wild-type animals relevant to these studies. Mice were maintained in the Longwood Medical Research Center facility in accordance with guidelines of the Committee on Animals of the Harvard Medical School (Boston, Mass).

**Diet Protocols**

Two separate experiments were performed with identical diet protocols but different methods of lesion analysis. In both experiments, 10 ldlr$^{-/-}$ C3$^{-/-}$ and 10 age- and sex-matched ldlr$^{-/-}$ C3$^{+/+}$ littermate control mice were recruited at 5 to 8 weeks of age. At day 0 (initiation of study, before diet) serum samples were collected from overnight-fasted individual mice by tail-vein bleeding. The mice were then fed ad libitum a semipurified D12108-based, 40% kcal lipid, 1.25% (wt/wt) cholesterol diet synthesized by Research Diets, Inc.\textsuperscript{23} After 15 weeks of diet, mice were fasted overnight and killed by ether inhalation, blood was collected for serum analyses by vena

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Total Cholesterol, mg/dL</th>
<th>VLDL Cholesterol, mg/dL</th>
<th>LDL Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ldlr$^{-/-}$ C3$^{-/-}$</td>
<td>1215±271*</td>
<td>628±137†</td>
<td>445±125‡</td>
<td>74±24§</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ldlr$^{-/-}$ C3$^{+/+}$</td>
<td>1265±221*</td>
<td>776±213†</td>
<td>359±115‡</td>
<td>62±21§</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Overnight fasting serum samples for cholesterol analysis were obtained after 15 weeks of cholesterol diet feeding. *P=0.84; †P=0.35; ‡P=0.29; §P=0.62

cava nicking, and the arterial tree was perfused with PBS (25 mL). Perfused aortas from exsanguinated mice were dissected from the aortic valve to the iliac bifurcation.

**Preparation and Analysis of Aortic Tissue**

In experiment No. 1, the proximal 1 cm of aorta starting from the aortic valve was cut with a scalpel and rapidly frozen in optimal cutting temperature embedding medium (OCT medium, Tissue-Tek). Serial 5-μm-thick cryostat sections were prepared, and every tenth section of the proximal 1000 μm of aorta was stained with oil-red O and counterstained with Wrights/Geimsa solution. To quantify cross-sectional area of lesions, digital photomicrographs of these sections were subject to image analysis with IMAGEPRO PLUS software (Media Cybernetics). The fractional area of the lesions, expressed as percentage of total sectional area of aortic wall, was calculated, as described.\textsuperscript{24} Adjacent cryostat sections were analyzed by immunohistochemistry, as described below. The remaining thoracic and abdominal aorta from each mouse in experiment No. 1 was fixed in 10% buffered formalin, stained with oil-red O, opened longitudinally, pinned out, and photographed with a digital camera to obtain images of lesions en face, as previously described.\textsuperscript{25} The percent surface area occupied by oil-red O–stained lesions viewed en face was determined with IMAGEPRO PLUS software.

In experiment No. 2, the aortic arches from mice were dissected from the aortic valve, separated from the remaining aorta, and frozen in OCT medium. Five micron-thick longitudinal-cryostat sections were prepared, which included greater and lesser curvatures of the aortic arch from the aortic root to beyond the takeoff of the left subclavian artery. These sections were stained with oil-red O and photographed with a digital camera attached to a microscope. A defined portion of the aortic arch was quantitatively analyzed in each section, consisting of a 3-mm segment of the lesser curvature.
defined proximally by the aortic root and distally by a perpendicular axis dropped from the distal side of the left common carotid artery origin.²⁴ Medial, intimal, and lipid-positive areas within this 3-mm stretch of aorta were calculated for each mouse by computerized image analysis with IMAGEPRO PLUS software.

**Immunohistochemical and Histochemical Analyses of Aortic Lesions**

In experiment No. 1, cross sections of proximal aorta were stained for C3 and IgG. Cryostat microtome cross sections (5 μm thick) were fixed in cold acetone (4°C, 7 minutes), rehydrated in PBS, and blocked with PBS, 1% hydroperoxide in PBS, and 10% normal goat serum. The sections were stained with horseradish peroxidase (HRP)–conjugated goat IgG specific for mouse C3 (ICN Pharmaceuticals), HRP-conjugated goat anti-mouse IgG F(ab')2 (ICN), and HRP-conjugated goat IgG (Jackson ImmunoResearch Laboratories) as isotype control. Staining was visualized by adding 3-amino-9-ethylcarbazole (AEC substrate kit (ABC Elite Peroxidase kit, Vector Laboratories) and counterstaining with Gill's No. 2 hematoxylin solution (Polysciences). To stain smooth muscle cells, alkaline phosphatase-conjugated monoclonal anti-smooth muscle actin antibody Clone 1A4 (Sigma, St Louis, Mo) was used, and the reaction was visualized by RED substrate (kit for alkaline phosphatase, Vector Laboratories). Collagens type I and III were stained with Picosiris red, as described, and the sections were analyzed by polarization microscopy.

**Serum Lipid and Immunoglobulin Analyses**

Overnight fasting blood from individual mice collected at day 0 (before diet) and after 15 weeks of special diet (after diet) was allowed to clot, and the serum fraction, after microcentrifugation, was stored at −20°C until specific assays were performed.

Serum lipoprotein profiles were determined by gel-filtration fast-performance liquid chromatography from 6 animals in each group and by enzymatic assays (Roche Diagnostics).

Total Ig levels were determined by isotype-specific ELISA (Southern Biotechnology Associates). The concentration (expressed in mg/dL) of the individual Ig isotype was calculated from linear regression analysis of a standard curve generated with isotype standards supplied with the ELISA kit.

Autoantibodies to specific epitopes of oxidized LDL were detected in serum samples from diet-fed mice. Titors of serum IgG and IgM specific for malondialdehyde (MDA)–LDL and 1-palmitoyl-2-5-oxovaleroyl-phosphatidylcholine–LDL were determined as described.

**Statistical Analysis**

Serum lipid and Ig data, as well as data from quantitative image analysis of immunohistochemically stained sections, were determined to be normally distributed (Prism software). For these data sets, the Student’s t test was used to analyze differences between the groups, and the data are expressed as mean±SEM. Data on lesional intimal area and lipid positive area, shown in Figure 3, were not uniformly normally distributed; therefore, the differences between ldlr−/−C3−/− and ldlr−/−C3+/+ groups were analyzed by the Mann-Whitney U test and expressed as the median. A value of P≤0.05 was considered significant for both types of analysis.

**Results**

Serum Lipid and Immunoglobulin Analyses

Analyses of serum lipids from the mice in these studies indicate that complement deficiency does not alter serum lipid content. After 15 weeks of high-fat/high-cholesterol diet, serum lipids were measured from individual mice collected at day 0 (before diet) and after 15 weeks of special diet (after diet) was allowed to clot, and the serum fraction, after microcentrifugation, was stored at −20°C until specific assays were performed.

**Figure 2.** Oil-red O staining of aortic atherosclerotic lesions in complement-deficient and control mice. Aortas from cholesterol-fed ldlr−/−C3−/− mice (top) and ldlr−/−C3+/+ (bottom) are shown. In experiment No. 1 (left), cross sections of the proximal ascending aorta were stained, and in experiment No. 2 (right), longitudinal sections of the aortic arch were stained. Arrows in left panels indicate corresponding lipid-rich and collagen-rich regions in C3-deficient and control animals, respectively.
diet, the mean total, very-low-density lipoprotein (VLDL), LDL, and HDL cholesterol levels in sera of ldlr<sup>/−/−</sup> C3<sup>+/−</sup> were not statistically different from ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice, as determined by gel-filtration fast-performance liquid chromatography (Table 1, Figure 1). Enzymatic assays of total, LDL, and HDL cholesterol also did not reveal significant differences between ldlr<sup>/−/−</sup> C3<sup>+/−</sup> and ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice (data not shown).

C3 deficiency in mice is associated with reduced IgG production in response to T-dependent antigens, but serum IgM and IgG levels are not significantly different in C3-null versus wild-type mice. Total serum Ig levels of C3-deficient mice did not differ from control mice at time 0 (before diet) or after diet (Table 2). Furthermore, we did not see significant differences between ldlr<sup>/−/−</sup> C3<sup>+/−</sup> and ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice (data not shown).

**Quantitative Analysis of Atherosclerotic Lesions**

After 15 weeks of cholesterol-diet feeding, both ldlr<sup>/−/−</sup> C3<sup>+/−</sup> and ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice had aortic atherosclerotic lesions with intimal proliferation and foam cells, although the fat content appeared greater in the lesions from complement-deficient mice (Figure 2). In experiment No. 1, the median en face aortic lesion area in descending and abdominal aorta in ldlr<sup>/−/−</sup> C3<sup>+/−</sup> was significantly greater than in ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice (Figure 3). The median cross-sectional area of the proximal aortic lesions, as a percentage of total arterial wall sectional area, was not significantly different between ldlr<sup>/−/−</sup> C3<sup>+/−</sup> and ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice.

In experiment No. 2, analysis of longitudinal sections of aortic arch revealed significantly greater lipid positive areas in ldlr<sup>/−/−</sup> C3<sup>+/−</sup> compared with ldlr<sup>/−/−</sup> C3<sup>+/+</sup>, whereas total intimal areas were not different between the 2 groups (Figure 3, bottom). These combined data indicate that C3 enhances lesional lipid content but not total lesional size. Medial areas were also not statistically different between the 2 groups (data not shown).

**Phenotypic Analysis of Atherosclerotic Lesions**

Lesional C3 and IgG depositions were detectable in proximal aortic cross sections from ldlr<sup>/−/−</sup> C3<sup>+/−</sup> mice, whereas IgG but not C3 was detectable in lesions from ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice (Figure 4). Both C3 and IgG were deposited in the lesional neointima and the subjacent media.

Cell and matrix content of aortic arch lesions appeared quantitatively different in ldlr<sup>/−/−</sup> C3<sup>+/−</sup> versus ldlr<sup>/−/−</sup> C3<sup>+/+</sup> mice (Figure 5), with more macrophages but less collagen and smooth muscle cells in the lesions of C3-deficient mice. Smooth muscle actin was evident in the region of early fibrous cap formation in control mice but not C3-deficient mice. Image analysis confirmed that macrophage content was significantly greater in lesions of C3-deficient mice compared with controls, and smooth muscle cells and collagen content was significantly greater in control mice (Table 3).

**Discussion**

A principal finding of the present study is that the extent of total aortic atherosclerosis, determined by lipid staining, was
greater in aortas from C3-deficient mice compared with controls. The differences were evident from analysis of en face preparations of descending thoracic and abdominal aorta and in longitudinal sections of aortic arch. In addition, the median lipid-positive area of proximal aortic cross sections was greater in C3-deficient versus control mice, but the differences between the experimental groups was not statistically significant.

Figure 4. C3 and immunoglobulin deposition in aortic atherosclerotic lesions. Cross sections of proximal ascending aorta from a C3-deficient mouse (top) or control mouse (bottom) in experiment No. 1 were stained with antibodies specific for C3 (left) or mouse Ig (right), as described in Methods. Sections shown are representative of all animals in the respective groups.

Figure 5. Macrophage, smooth muscle cell, and collagen content of aortic arch atherosclerotic lesions in complement-deficient and control mice. Aortic arch sections from 2 complement-deficient mice (Nos. 483 and 485) and 2 control mice (Nos. 806 and 803) in experiment No. 2 were stained for smooth muscle actin (top 4 panels), macrophages (Mac 3) (middle 4 panels), and collagen (bottom 4 panels), as described in Methods. Approximately the same areas of each lesion are shown for macrophage and smooth muscle cell stains, and these areas are typical of lesions quantitatively analyzed in Table 3. Smooth muscle cell staining in the position of an early fibrous cap in control mice is indicated by arrows.
A second important finding in this study was the difference in lesion phenotype seen in C3-deficient versus control mice. Macrophages were more abundant in lesions from C3-deficient mice, whereas collagen and smooth muscle cells were more abundant in the control mice. Taken together with the lesion lipid staining, these data support the conclusion that in the absence of C3, lesions remain predominantly lipid and foam cell rich, and lesion maturation, indicated by smooth muscle cell migration or proliferation and the deposition of fibrous matrix, is impaired. One implication of this finding is that complement activation promotes changes that are associated with plaque stability. Our data from the 15-week time point do not distinguish the possibilities that C3 deficiency results in a complete block versus slower kinetics of lesion maturation, but both possibilities are of significance in the natural history of atherosclerotic disease.

The C3-derived peptide C3ades-Arg is reported to regulate triglyceride synthesis and apobetalipoprotein.\(^{27}\) However, the importance of C3ades-Arg in systemic lipid metabolism remains controversial.\(^{28}\) We did not find any differences in fasting lipoprotein profiles between C3-deficient and control mice, consistent with a previous study,\(^{29}\) indicating that the complement system does not have a major impact on circulating lipoprotein levels in mice. We also did not find significant differences in serum Ig levels between the experimental groups. The serum Ig and lipid data support the conclusion that the differences we did observe in the extent and phenotype of lesions in C3-deficient and control mice is most likely attributable to direct local effects of complement activation within the lesions. Consistent with this conclusion is the fact that there was IgG and C3 deposition within aortic lesions of the control animals in this study and IgG but not C3 deposition in the lesions of C3-deficient mice.

Byproducts of complement activation are known to have many biological properties that could influence the development of atherosclerotic lesions. Local complement activation may induce cell lysis and generate at least some of the cell debris found in the necrotic core of advanced lesions.\(^{10}\) This would be consistent with our finding of more macrophages in lesions from C3-deficient mice. Sublytic assembly of C5b-9 on smooth muscle cells are reported to induce cell activation and proliferation.\(^{30}\) In support of this, we found fewer smooth muscle cells in the lesion of C3-deficient mice compared with controls.

Previous studies of atherosclerosis in complement-deficient animals have focused on terminal lytic pathway components and are contradictory. C6-deficient rabbits seemed to be protected from lesion development,\(^{19}\) whereas aortic root lesion size in C5-deficient ApoE-null mice was not different from control ApoE mice.\(^{20}\) The latter mouse study did not examine more distal regions of the aorta, nor did it examine lesion phenotype. We also did not see statistically significant differences in lipid-positive area of proximal aortic cross sections in complement-deficient and control mice, but we did see differences when aortic arch or descending aorta were analyzed. Although activation of the terminal lytic pathway requires C3, there are likely to be effects of C3 on atherosclerosis that are independent of the terminal pathway.

In conclusion, we have demonstrated that the progression of atheromas from foam cell–rich and lipid-rich lesions to lesions with prominent smooth muscle cells and collagen depends in part on the presence of an intact complement system. Our findings indicate that complement activation should be considered when evaluating the mechanisms and prognostic significance of other inflammatory parameters associated with atherosclerosis, including CRP, and humoral immune responses.

### Acknowledgments

This work was supported by National Institutes of Health grants HL56985 (Drs Lichtman and Buono), HL48743 (Dr Lichtman), HL56989 (Dr Witztum), and AI36389 (Dr Carroll).

### References


### Table 3. Quantitative Analysis of Cell and Matrix Content of Aortic Arch Lesions in Idlr/−/C3−/− and Idlr/−/C3+/+ Mice

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Macrophages, % Intimal Area</th>
<th>Smooth Muscle Cells, % Intimal Area</th>
<th>Collagen, % Intimal Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idlr/−/C3−/−</td>
<td>8.24±1.36*</td>
<td>0.06±0.05†</td>
<td>0.52±1.26‡</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idlr/−/C3+/+</td>
<td>5.9±1.63*</td>
<td>0.92±0.32†</td>
<td>11±10.43‡</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P=0.003; †P=0.0000003; ‡P=0.008.


Influence of C3 Deficiency on Atherosclerosis
Chiara Buono, Carolyn E. Come, Joseph L. Witztum, Graham F. Maguire, Philip W. Connelly, Michael Carroll and Andrew H. Lichtman

Circulation. 2002;105:3025-3031; originally published online June 3, 2002;
doi: 10.1161/01.CIR.000019584.04929.83
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 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/105/25/3025

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/