Membrane Type 1 Matrix Metalloproteinase Expression in Human Atherosclerotic Plaques
Evidence for Activation by Proinflammatory Mediators

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Background—Matrix metalloproteinases (MMPs) are expressed in atherosclerotic plaques, where in their active form, they may contribute to vascular remodeling and plaque disruption. In this study, we tested the hypothesis that membrane type 1 MMP (MT1-MMP), a novel transmembrane MMP that activates pro-MMP-2 (gelatinase A), is expressed in human atherosclerotic plaques and that its expression is regulated by proinflammatory molecules.

Methods and Results—MT1-MMP expression was examined in normal and atherosclerotic human arteries by immuno-cytochemistry with specific antibodies. MT1-MMP expression in human saphenous vein–derived smooth muscle cells (SMCs) maintained in tissue culture was determined under basal conditions and in response to proinflammatory molecules (interleukin [IL]-1α, tumor necrosis factor [TNF]-α, and oxidized LDL [ox-LDL]) by use of Northern blot and ribonuclease protection assays for mRNA, Western blot and immunoprecipitation for protein, and gelatin zymography for catalytic activity. Medial SMCs of normal vessel wall expressed MT1-MMP. In atherosclerotic arteries, MT1-MMP expression was noted within the complex atheroma colocalizing with SMCs and macrophages (Mφ).

Cultured SMCs constitutively expressed MT1-MMP mRNA and protein, which increased 2- to 4-fold over control in a time-dependent manner within 4 to 8 hours of exposure to IL-1α, TNF-α, and ox-LDL (thiobarbituric acid–reactive substances, 13.4 nmol/mg LDL protein), whereas native LDL had no effect. Flow cytometry revealed MT1-MMP expression by human monocyte-derived Mφ, which increased 3.8-fold over baseline within 6 hours after exposure to 10 ng/mL TNF-α.

Conclusions—This study demonstrates that MT1-MMP, an activator of pro-MMP-2, is expressed by SMCs and Mφ in human atherosclerotic plaques. Furthermore, proinflammatory molecules upregulate MT1-MMP expression in vascular SMCs and Mφ. Thus, activation of SMCs and Mφ by proinflammatory molecules may influence extracellular matrix remodeling in atherosclerosis by regulating MT1-MMP expression. (Circulation. 1999;99:3103-3109.)

Key Words: metalloproteinases ■ cells ■ proteins ■ lipoproteins

Matrix metalloproteinases (MMPs) are a family of structurally related proteases that are capable of degrading virtually all components of the extracellular matrix (ECM). A number of MMPs are expressed by smooth muscle cells (SMCs) and macrophages (Mφ) in atherosclerotic and injured vessels, where they may participate in vascular remodeling, SMC migration, neointima formation, and plaque disruption. The MMPs are secreted as zymogens, requiring activation by limited proteolysis, and their activity is inhibited by naturally occurring inhibitors called tissue inhibitors of MMPs (TIMPs). Unlike other secreted MMPs, MMP-2 activation does not occur through proteolysis by serine proteases but rather through a cell-surface–associated MMP activator, although thrombin and integrin-α,β may also activate pro-MMP-2. Several membrane-bound MMPs have recently been identified with membrane type 1 MMP (MT1-MMP) or MMP-14, representing the prototypical form of membrane-bound activator of pro-MMP-2. Expression of MT1-MMP in human atherosclerotic plaques has not been reported.

In the present study, we tested the hypothesis that MT1-MMP is expressed in human atherosclerotic plaque and that proinflammatory molecules regulate its expression in vitro.

Received August 12, 1998; revision received March 31, 1999; accepted March 31, 1999.
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Presented at the 47th annual scientific sessions of the American College of Cardiology, Atlanta, Ga, March 29–April 1, 1998; and published in abstract form (J Am Coll Cardiol. 1998;31:419A).
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Methods

Materials
Purified mouse monoclonal antibodies to human MT1-MMP, MMP-2, and TIMP-2 were purchased from Oncogene Research Products. Peroxidase-conjugated rabbit anti-mouse IgG was obtained from Zymed Laboratories. SMC marker HHF35, M6 marker CD68 (PG-M1), and mouse nonspecific IgG (used as a negative control) were purchased from Dako. Goat IgG used to block nonspecific binding in the flow cytometric analysis was purchased from Santa Cruz Biotechnology. Phycocerythrin (PE)-conjugated anti-mouse goat IgG was purchased from Caltag Laboratories. All tissue culture media and supplements were purchased from Gibco-BRL. Fetal calf serum (FCS) was from Hyclone Laboratories. Human cytokine media and supplements were purchased from GIBCO-BRL. Fetal bovine serum (FBS) and knockout serum replacement (KSR) were purchased from Cambrex. Fetal calf serum (FCS) was from Hyclone Laboratories. Human cytokine media and supplements were purchased from GIBCO-BRL. Fetal bovine serum (FBS) and knockout serum replacement (KSR) were purchased from Cambrex. Fetal bovine serum (FCS) was from Hyclone Laboratories. Human cytokine media and supplements were purchased from GIBCO-BRL. Fetal bovine serum (FBS) and knockout serum replacement (KSR) were purchased from Cambrex. 

Immunocytochemical Staining
Sections of normal and atherosclerotic arteries were subjected to immunohistochemical staining for MT1-MMP and MMP-2. Staining for SMCs and M\(\alpha\) were performed with cell-type-specific antibodies. A human lung adenocarcinoma specimen, which has previously been shown to express MT1-MMP,\(^1\) was used as a positive control. Tissue specimens were deparaffinized with xylene followed by immersion in graded ethanol. They were washed 3 times for 5 minutes each in PBS and blocked with 3% rabbit serum in PBS for 30 minutes. Specimens were then exposed to primary antibody against MT1-MMP (5 \(\mu\)g/mL) overnight at 4°C. Controls included nonspecific IgG as negative control (5 \(\mu\)g/mL) instead of primary antibody and use of PBS instead of secondary antibody. After they were washed in PBS, specimens were incubated with biotinylated rabbit anti-mouse IgG for 30 minutes in a humidified chamber at room temperature. Specimens were then washed with PBS and stained with horseradish peroxidase-conjugated streptavidin for 30 minutes. Specimens were finally incubated with substrate solution for 1 to 15 minutes and counterstained with dilute hematoxylin.

Atherosclerotic plaques were immunostained with human TIMP-2–specific antibody by use of a similar technique.

Isolation and Culture of Human Vascular SMCs and Monocyte-Derived M\(\alpha\)
SMCs were isolated from human saphenous vein with type II collagenase, as previously described.\(^17\) Cells after 3 passages were used throughout the experiments and were studied at confluence in all treatment conditions. Preparation and characterization of ox-LDL were performed essentially as described previously.\(^18,19\) Cells were treated with cytokines (10 ng/mL) or ox-LDL (100 \(\mu\)g/mL), as previously described.\(^20\) All reagents in our tissue culture studies were verified for the absence of endotoxin by a commercially available assay kit (BioWhittaker) that has a sensitivity detection level of 1 pg/mL. The final concentration of endotoxin in lipoprotein preparations was <20 pg/mL of the culture medium used.

Peripheral blood monocytes were isolated as described previously.\(^20\) Monocyte-derived M\(\alpha\) were cultured in RPMI 1640 containing 10% FCS, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 0.25 \(\mu\)g/mL amphotericin B for 5 days and then starved in the culture medium without FCS but with 0.1% low endotoxin BSA (Sigma). Experiments were performed in the starvation medium with or without TNF-\(\alpha\) (10 ng/mL).

Northern Blotting and Ribonuclease Protection Assays
Total cellular RNA was isolated by lysis of SMCs in guanidinium isothiocyanate, phenol-chloroform extraction, and ethanol precipitation.\(^21\) Each RNA preparation (20 \(\mu\)g) was denatured and electrophoresed through a 1.2% formaldehyde agarose gel, after which each preparation was blotted onto nylon filters and subjected to ultraviolet (UV) cross-linking. Filters were hybridized with isolated and radiolabeled MT1-MMP–specific cDNA probe.\(^18\) The blots were washed, autoradiographed, and then rehybridized with a \(\beta\)-actin cDNA probe as an internal control. Quantitative results of the assays were obtained by densitometry of autoradiograms. Ribonuclease protection assays on the total RNA were performed with radiolabeled anti-sense MT1-MMP and \(\beta\)-actin sequences by use of a commercially available kit (Ambion).

Immunoblotting
Extracts of partially purified plasma membrane fractions of SMCs treated with IL-1\(\alpha\), TNF-\(\alpha\), ox-LDL, or not were isolated as described previously.\(^22\) Briefly, SMCs were suspended in ice-cold lysis buffer (0.1 mmol/L PMSF, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 mmol/L NaCl, and 5 mmol/L Tris-HCl; pH 7.4). Mechanical disruption of the cells was performed by Polytron homogenizer, and the lysates were centrifuged at 40,000g for 30 minutes at 4°C. The crude pellet was washed twice with ice-cold suspension buffer and then centrifuged at 3000g for 10 minutes. The pellet was discarded, and the supernatant was centrifuged again at 40,000g for 45 minutes at 4°C. The resulting pellet containing the enriched membrane preparation was then resuspended at a protein concentration of 2 mg/mL in a buffer containing HEPEs, EGTA, EDTA, and NaCl; snap-frozen, and stored at −70°C.

Proteins of SMC membranes (50 \(\mu\)g) and known molecular weight markers were separated by SDS-PAGE, transferred onto Western polyvinyl difluoride membranes, and incubated overnight at 4°C with blocking solution (5% skim milk in PBS). Purified mouse monoclonal antibodies (10 \(\mu\)g of IgG per mL) to human MT1-MMP were incubated with the blots overnight at 4°C in PBS buffer containing 0.1% Tween 20.\(^23\) The blots were washed twice with PBS buffer and then treated with rabbit anti-mouse antibody (1:4000 dilution) coupled to horseradish peroxidase. Immunodetection was accomplished with an enhanced chemiluminescence kit from Amersham.

Immunoprecipitation and Gelatin Zymography
MT1-MMP immunoprecipitation was performed in the presence of protease inhibitor cocktail (Boehringer Mannheim), as previously described.\(^23\) Equal amounts of extracts of partially purified plasma membrane fractions of SMCs, untreated or treated with IL-1\(\alpha\) or ox-LDL, were incubated with purified mouse monoclonal antibodies to either human MT1-MMP or an irrelevant protein. Antigen-antibody complexes were precipitated with protein G and A–coupled agarose beads (Oncogene Research Products) by centrifugation. Equal amounts of the supernatants were added to culture media harvested from human SMCs containing pro-MMP-2 and assayed for gelatinolytic activity essentially as described previously.\(^24\) Proteins were electrophoresed in the presence of SDS in discontinuous 10% SDS-PAGE containing 1 mg/mL gelatin. Gels were processed to renature the protein by exchanging SDS to Triton X-100 (2 changes of 2.5% Triton X-100 for a total of 30 minutes). Gels were subsequently incubated for 18 hours at 37°C in 50 mmol/L Tris-HCl, pH 7.4, containing 10 mmol/L CaCl\(_2\), and 0.05% Brij 3 and stained with Coomassie brilliant blue G (Sigma), followed by destaining in 5% methanol and 7% acetic acid.

Flow Cytometric Analysis of Cultured Human Monocyte-Derived M\(\alpha\)
Human cultured M\(\alpha\) grown in absence or presence of TNF-\(\alpha\) were harvested by treating the culture with cold PBS for 30 minutes and then scraping the cells from the plastic. The cells were pelleted by centrifugation and incubated with 20 \(\mu\)g of goat IgG in PBS/0.1% sodium azide on ice for 10 minutes. Primary monoclonal antibody (0.5 \(\mu\)g per sample to a total volume of 50 \(\mu\)L) was added to cells, which were then incubated on ice for 30 minutes. After 2 washes with PBS containing 1% FCS/0.1% sodium azide, cells were incubated with saturating concentrations of PE-conjugated goat anti-mouse IgG for 30 minutes at 4°C for 10 minutes. After 2 more washes, cells were fixed with 1% paraformaldehyde in PBS. Anal-
ysis was performed with FACScan (Becton Dickinson). Cell populations were gated according to forward and side scattering. Results were plotted as intensity of fluorescence versus cell number.

Data Analysis
Intensities of experimental bands from the RNA and protein assays were measured by computer-assisted densitometry. Results are expressed as mean±SEM. Statistical analyses were performed by Student’s t test to determine the significance of change in the densitometric measurements. A significant difference was considered for probability value ≤0.05.

Results
Localization of MT1-MMP in Normal and Atherosclerotic Human Arteries
Normal human coronary arteries showed abundant MT1-MMP immunoreactivity that colocalized with MMP-2 antigen staining within the medial SMC (Figure 1; a, b, and c). Human atherosclerotic plaques contained MT1-MMP and MMP-2 proteins in the media underlying fibrous and lipid-rich regions (Figure 1; d, e, and f). Both MT1-MMP and MMP-2 proteins were also detectable in Mϕ of the fibrous plaques (Figure 1; g, h, and i). Mouse nonspecific IgG used in place of primary antibody showed no background or nonspecific staining (Figure 1j) in atheromatous plaque. Human lung adenocarcinoma specimens, which have previously been shown to express MT1-MMP, served as a positive control, as shown in the inset of Figure 1j. Lipid-rich plaques, as shown in Figure 1k, exhibited expression of MT1-MMP that was mostly localized to Mϕ, as determined by counting cells in defined areas under a light microscope. Lipid-rich plaques also exhibited expression of MMP-2 (Figure 1l). In addition, abundant TIMP-2 expression was observed in atherosclerotic plaques (Figure 1; m and n).

Augmented Expression of MT1-MMP mRNA in Stimulated SMCs
Northern blotting showed that SMCs contained a single mRNA species of 4.5 kb (Figure 2), a size similar to MT1-MMP mRNA observed in normal lung tissue and tumor cells.15 These assays also revealed that exposure of cultured SMCs to IL-1α, TNF-α, or ox-LDL resulted in the accumulation of MT1-MMP mRNA (Figures 2, 3, and 4). Both IL-1α and ox-LDL caused a time-dependent progressive increase in steady-state levels of MT1-MMP mRNA in stimulated SMCs. MT1-MMP mRNA levels increased within 4 hours of exposure to IL-1α, reached a peak level of ≈4-fold above control by 6 hours, and remained elevated for ≥12 hours (Figure 3). The time course for the induction of MT1-MMP mRNA in response to ox-LDL appeared similar to IL-1-α. MT1-MMP mRNA levels increased (2.5-fold), peaked at ≈8 hours, and remained elevated for ≥16 hours (Figure 4).

Increased Expression of MT1-MMP Protein in Stimulated SMCs
To establish that SMCs expressed MT1-MMP mRNA translated into immunoreactive protein, we performed SDS-PAGE coupled with immunoblotting on the plasma membrane extracts of unstimulated and stimulated SMCs. SMCs constitutively expressed a membrane-associated protein that reacted with human MT1-MMP–specific antibody (Figure 5). Plasma membrane extracts derived from cells that had been stimulated with IL-1α, TNF-α, or ox-LDL exhibited increased immunoreactive MT1-MMP. The IL-1α–mediated increase in MT1-MMP mRNA correlated with an ≈3-fold increase in MT1-MMP protein levels in SMC membranes (Figure 5). Treatment of cells with ox-LDL also increased (2-fold) the level of MT1-MMP proteins in SMC membrane extracts.

Stimulation of MT1-MMP Enzymatic Activity in SMCs
To examine whether increased levels of MT1-MMP mRNA and immunoreactive protein correspond to augmented enzymatic activity, we performed SDS-PAGE gelatin zymography on plasma membrane extracts of SMCs stimulated with IL-1α or ox-LDL. Incubation of medium conditioned by human SMCs that contained pro-MMP-2 with plasma membrane extracts derived from cells that had been stimulated with IL-1α or ox-LDL increased the proteolytic conversion of 72-kDa pro-MMP-2 to new gelatinolytic bands of 70 and 68 kDa, corresponding to the processed active MMP-2 (Figure 6). Stimulation of SMCs with TNF-α also significantly increased the levels of membrane-associated MT1-MMP enzymatic activity (data not shown). Purified mouse monoclonal antibody to human MT1-MMP immunoprecipitated a 64-kDa protein of the size of MT1-MMP from the membrane extracts of unstimulated and stimulated SMCs (data not shown). Membrane-bound pro-MMP-2 proteolytic processing activity was reduced in membrane extracts after immunodepletion of MT1-MMP with the specific antibody (Figure 6).

Increased Expression of MT1-MMP Protein in Stimulated Mϕ
In the basal state, Mϕ expressed low amounts of MT1-MMP, with 14.3±3.9% of cells showing a positive event compared with the background level of 4.7±2.4% of cells. Exposure to TNF-α for 6 hours increased the number of positive cells to 41.4±0.3% of the cell population (Figure 7).

Discussion
In this study, we have demonstrated that MT1-MMP, a membrane-bound MMP that activates pro-MMP-2, is expressed in medial SMCs and to a lesser extent in adventitia of normal and atherosclerotic coronary arteries. This study also demonstrates MT1-MMP expression by SMCs and Mϕ in lipid-rich atherosclerotic plaques. The in vitro data from this study show that human saphenous vein–derived SMCs constitutively express MT1-MMP along with pro-MMP-2 and that proinflammatory molecules (IL-1α, TNF-α, and ox-LDL) augment MT1-MMP expression, leading to increased activation of pro-MMP-2. This suggests a functional interaction between MT1-MMP and MMP-2 in SMC-mediated vascular remodeling in normal and atherosclerotic human arteries.

Activation of SMCs and Mϕ by proinflammatory molecules generated in response to atherogenic stimuli appears to occur during various stages of atherosclerosis. Several recent studies indicate that ox-LDL may promote this process.25–27 We found that exposure of cultured SMCs to proinflamma-
tory cytokines and ox-LDL alters appreciably the steady-state levels of MT1-MMP mRNA. The augmented MT1-MMP mRNA correlated with increased plasma membrane-associated immunoreactive protein and catalytic function to precursor MMP-2, as demonstrated by Western blotting and gelatin zymography. These results provide a possible mechanism underlying the previous findings showing that IL-1– or TNF-α–stimulated human saphenous vein SMCs produce significantly increased levels of active MMP-2. The results of our studies raise the intriguing possibility that ox-LDL, directly or by inducing activators such as cytokines, may influence remodeling of the ECM in atherosclerosis. Previous studies showing that reactive oxygen species can promote activation of MMPs argue in favor of the concept that proinflammatory cytokines or ox-LDL mediate the activation of MT1-MMP by generating highly reactive oxygen species. Additional studies are required to test these possibilities.

MT1-MMP is a 64-kDa protein that contains a single transmembrane domain with the catalytic site positioned on the exterior surface of the cell. MT1-MMP belongs to a family of cell-bound proteins that specifically activate pro-MMP-2, which, unlike other MMPs, is not activated by
plasmin and other serine proteases. Pro-MMP-2 binds to MT1-MMP and becomes activated through limited proteolysis by MT1-MMP. The properties of MT1-MMP are consistent with the membrane localization and inhibition profiles attributed previously to the cell surface–associated MMP-2 activator. Membrane lysates of vascular SMCs induced the conversion of pro-MMP-2 to the fully activated (68 kDa) protein through the intermediate 70-kDa form. This catalytic conversion corresponded to IL-1α–, TNF-α–, or ox-LDL–induced levels of MT1-MMP mRNA and immunoreactive protein. Additionally, the affinity-purified anti–MT1-MMP antibody specifically blocked a substantial portion of the membrane-associated proteolytic activity that catalyzed the conversion of 72-kDa pro-MMP-2 to MMP-2. Because MT1-MMP belongs to a family of enzymes including ≥4 members, the residual activity that partially activates the conversion of pro-MMP-2 to MMP-2 after immunodepletion of MT1-MMP in our assays may be due to other members of the family or to related enzymes present in SMC membrane extracts.

Recent studies have suggested a unique mechanism by which MT1-MMP activates pro-MMP-2. MT1-MMP–induced activation of pro-MMP-2 appears to involve the formation of a complex with TIMP-2, which in turn can form a ternary complex of MT1-MMP/TIMP-2/pro-MMP-2. In the present study, we have also demonstrated immunohistochemical evidence for TIMP-2 expression in Mφ-rich regions of human atherosclerotic plaque. Galis et al previously showed evidence of the presence of active MMP-2 in human atheroma using in situ zymography. Thus, all 3 components of the ternary complex necessary for the generation of active MMP-2 coexist in the atherosclerotic plaque. Additionally, recent evidence suggests that MT1-MMP is also capable of directly degrading ECM. The exact functional significance of MT1-MMP expression in atherosclerotic plaque demonstrated in the present study remains to be clarified.
findings, however, provide evidence that coexpression of MT1-MMP and MMP-2 in SMCs could play a critical role in normal vascular homeostasis and may also contribute to abnormal matrix turnover and remodeling in atherosclerotic plaques. The presence of an activator of pro-MMP-2 on the plasma membrane of SMCs or Mφ may serve to localize matrix degradation near the cell surface, permitting a focal and controlled dissolution of ECM.

The results of our studies suggest that inflammatory activation of MT1-MMP may contribute to the enhanced local matrix degradation in atherosclerotic plaques. Experimental studies with MMP inhibitors have demonstrated inhibition of neointima formation in a double-injury–restenosis model in the pig, suggesting a potentially critical role for MMPs in vascular SMC migration and accumulation as well as collagen gene expression after vascular injury.11 Similarly, focal expression of several MMPs, including MMP-2, in rupture-prone regions of human atherosclerotic plaque, as well as the previously demonstrated ability to induce collagen breakdown in vitro and in vivo, provides support for the role of MMPs in plaque disruption and consequent acute coronary thrombosis.8,9,31 Preferential expression of MMP-2 and TIMP-2 has been demonstrated in areas of superficial plaque erosions and endothelial desquamation in plaque erosion.32 An improved understanding of the factors that regulate the activity of MMPs in general and MMP-2 in particular should provide new directions for the development of novel therapeutic interventions to prevent atherosclerotic plaque growth, erosion, and rupture or restenosis after angioplasty.

Acknowledgments
This research work was supported by funds from the National Heart, Lung, and Blood Institute (grants HL-51980 and HL-58555 to Dr Rajavashisth) and by a generous grant from the Grand Foundation of Los Angeles, Calif (Dr Shah). Dr Xu was supported by the Dunitz family fellowship award during this research. Dr Jovinge was supported by a fellowship from the Johann and Throne Holst and the Swedish Heart and Lung Foundations. We thank Dr Judith Berliner for providing native and oxidized LDL preparations, Alan Wagner and Leslie Drake for human monocyte-derived Mφ, and Sangeetika Tripathi for expert technical assistance. The authors thank Maria Muszynski, DVM, of the Brigham and Women’s Hospital, Boston for help with human SMC cultures.

References

Figure 5. Immunoblotting of partially purified SMC membrane extracts (50 μg) with MT1-MMP antibody from untreated cells (Control) and cells treated with IL-1α (10 ng/mL), TNF-α (10 ng/mL), native LDL (n-LDL; 100 μg/mL; TBARS, 0.2 nmol/mg of LDL protein), or ox-LDL (100 μg/mL; TBARS, 13.4 nmol/mg of LDL protein) for 24 hours. This blot shows representative of 3 separate experiments. Bottom graph displays densitometric analyses of immunoblots showing increases in MT1-MMP protein levels by IL-1α, TNF-α, n-LDL, or ox-LDL. Values are mean±SEM of 3 separate experiments. *P<0.05, **P<0.02 compared with untreated control.

Figure 6. Increased proteolytic processing of pro-MMP-2 from media conditioned by unstimulated human SMCs and by plasma membrane preparations of SMCs stimulated with either ox-LDL (100 μg/mL; TBARS, 13.4 nmol/mg of LDL protein) or IL-1α (10 ng/mL) for 24 hours. Note that immunoprecipitation of MT1-MMP by specific antibody reduces processing of pro-MMP-2 to MMP-2. Positions of molecular weight markers are indicated in kDa.

Figure 7. Flow cytometric analysis of human Mφ with purified mouse monoclonal antibody to human MT1-MMP. A, Untreated; B, 6 hours after TNF-α treatment. Plots represent distribution of arbitrary fluorescence units (on abscissas, on a logarithmic scale) as a function of cell number (on ordinates). Insets represent median value of distribution ±SEM. Panels shown represent 1 of 3 separate experiments.


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Circulation. 1999;99:3103-3109
doi: 10.1161/01.CIR.99.24.3103

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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