Inhibition of Late Vein Graft Neointima Formation in Human and Porcine Models by Adenovirus-Mediated Overexpression of Tissue Inhibitor of Metalloproteinase-3

Sarah J. George, PhD; Clinton T. Lloyd, FRCS; Gianni D. Angelini, FRCS, MD; Andrew C. Newby, PhD; Andrew H. Baker, PhD

Background—Autologous saphenous vein coronary artery bypass graft surgery is complicated by late graft failure due to neointima formation and subsequent atherosclerosis. Growth factors and metalloproteinases (MMPs) act in concert to promote neointima formation. Tissue inhibitor of metalloproteinase-3 (TIMP-3), an extracellular matrix–associated MMP inhibitor, uniquely promotes apoptosis of isolated vascular smooth muscle cells. Here, we overexpressed TIMP-3 at the luminal surface of human saphenous veins before organ culture and in pig saphenous veins before interposition grafting into carotid arteries in vivo to assess neointima formation.

Methods and Results—In both models, high TIMP-3 immunoreactivity occurred in the luminal and upper medial extracellular matrix after adenovirus delivery. MMP activity measured by in situ zymography was reduced throughout the veins, confirming a bystander effect. By use of 3 independent techniques, apoptosis levels in the neointima and medial layer were significantly elevated by TIMP-3 overexpression. Neointima formation was reduced by 84% in 14-day human organ cultures and by 58% in 28-day pig vein grafts (both \( P < 0.05 \)). In contrast, TIMP-2 overexpression had no effect on neointima formation in vivo.

Conclusions—Our results highlight the potential therapeutic benefit for TIMP-3 overexpression to reduce neointima formation associated with late vein graft failure. (Circulation. 2000;101:296-304.)

Key Words: metalloproteinases • genes • viruses • grafting • neointima

Coronary artery bypass graft surgery is initially effective in relieving the symptoms of angina and prolongs survival. Arterial conduits are preferred, but the requirement for multiple grafts results in the use of autologous saphenous vein. Late vein graft failure occurs as a result of neointima formation and superimposed atherosclerosis, resulting in a 30% to 50% failure rate within 10 years.1 Because of the lack of effective pharmacological interventions for treatment of late vein graft failure, we have focused on the possibility of using a gene-based therapy.

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Matrix-degrading matrix metalloproteinases (MMPs) have been implicated in the progression of vascular diseases.2–5 Together, MMPs can degrade all components of the extracellular matrix (ECM),6 and dysregulation of MMPs promotes migration and/or proliferation of vascular smooth muscle cells (SMCs). MMP activity is regulated by binding of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), a family of 4 members,7–10 to both pro-MMPs and active forms of MMPs.11–15

Recently, the role of MMPs in vascular pathologies has been substantiated by TIMP overexpression studies. Overexpression of TIMP-1 or -2 inhibits neointima formation in the rat model of angioplasty restenosis and in an in vitro human model of vein graft neointima formation.16–20 Overexpression of TIMP-3, but not TIMP-1 or -2, promotes apoptosis of SMCs.21 This novel function prompted us to define whether TIMP-3 would be useful as a novel gene therapeutic approach for prevention of vein graft neointima formation.

Methods

Materials

Unless stated, all chemicals were obtained from Sigma. Media, antibiotics, and FCS were purchased from Gibco/BRL; nucleotides from Boehringer Mannheim; and enzymes from Promega. Replication-defective recombinant adenoviruses RAdlacZ (reporter gene), RAdTIMP-2 (human TIMP-2), and RAdTIMP-3 (human TIMP-3) have been described elsewhere.21

Adenovirus-Mediated Infection of Human Saphenous Vein and Subsequent Organ Culture

Luminal delivery of adenoviruses to human saphenous vein was performed as described.18 Briefly, 4- to 6-cm segments were cannu-
lated, and the luminal surface was exposed to adenovirus at 1.2×10^10 pfu/mL at physiological pressure for 1 hour. Segments of vein were cultured for 14 days in RPMI 1640 medium containing 30% FCS and antibiotics.

**Infection of Pig Saphenous Vein and Graft Procedure**

The saphenous vein from each hind limb of 20- to 30-kg Landrace pigs was surgically exposed, and the side branches were ligated. The vein was placed in sterile medium, cannulated, and flushed and infused with vehicle (10 mmol/L Tris-HCl, 125 mmol/L NaCl, 1 mmol/L MgCl₂, pH 7.4) or adenovirus at 2.5×10^10 pfu/mL without distension for 30 minutes at room temperature. Heparin (100 IU/kg) was administered systemically, and the vein was anastomosed as an end-to-end interposition graft into each common carotid artery. The pigs were maintained on a normal chow diet for 7 or 28 days before explantation.

**Localization of Transgene Production and Other Immunocytochemical Procedures**

For analysis of β-galactosidase expression, explanted grafts were stained with X-gal stain [100 mmol/L sodium phosphate, pH 7.3 (77 mmol/L Na₂HPO₄, 23 mmol/L NaH₂PO₄), 1.3 mmol/L MgCl₂, 3 mmol/L K₂Fe(CN)₆, 3 mmol/L K₃Fe(CN)₆, and 1 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopuransidase)] for 16 hours at 37°C. Staining was photographed en face, and the veins were then cross-sectioned and stained with nuclear fast red stain.

Immunocytochemistry was performed with a monoclonal anti–human TIMP-3 antibody (TCS Biologicals). Briefly, frozen sections were incubated with antibody (1 µg/mL) or isotype-matched control IgG for 18 hours at 4°C. Immunoreactivity was visualized with Extravidin peroxidase and diaminobenzidine staining. Local overexpression of TIMP-3 in pig vein grafts was quantified by image analysis within 200 µm from the luminal surface. SMCs and endothelial cells were identified by immunostaining with a smooth muscle cell actin for SMCs (human and pig tissue) or QBend10 human (Dako) and DBA lectin (pig vein grafts; Vector Laboratories) for endothelial cells.

**Evaluation of MMP Inhibition**

Veins were analyzed for gelatinolytic activity by gelatin in situ zymography as described previously.23 Briefly, frozen sections (4 sections per sample, n=3 grafts per group) were coated with LM-1 photographic emulsion (Amersham International) diluted 1:2 with incubation medium (50 mmol/L Tris-HCl, 50 mmol/L NaCl, 10 mmol/L CaCl₂, 0.05% [wt/vol] Brij 35, pH 7.6). After incubation overnight (human) and for 4 hours (pig) at 37°C, slides were developed and fixed. Controls included 20 mmol/L EDTA or 200 mmol/L MMP inhibitor Ro 31-9790 (Roche Diagnostics Ltd).

**Detection of Apoptosis**

Three independent methods were used to evaluate apoptosis in human veins (n=6) and pig vein grafts (n=4 per group). First, in situ end-labeling (ISEL) was performed. In brief, paraffin sections were washed twice in 1×TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and incubated in 50 mmol/L Tris-HCl, pH 7.2, 10 mmol/L MgSO₄, 0.1 mmol/L dithiothreitol, 0.01 mmol/L each of dATP, dCTP, and dGTP, 0.01 mmol/L biotin-dUTP, and 8 U/mL of DNA polymerase I (Klenow). Incorporated biotin-dUTP was detected with Extravidin peroxidase and counterstained with hematoxylin. Second, terminal dUTP nick end-labeling (TUNEL) was performed as described.24 Sections were incubated in 100 mmol/L cacodylate (pH 6.4), 1 mmol/L CoCl₂, 0.1 mmol/L dithiothreitol, 0.01 mmol/L each of dATP, dCTP, and dGTP, and dOTP, 0.01 mmol/L biotin-dUTP, and 8 U/mL terminal deoxynucleotidyl transferase at 37°C for 60 minutes. Third, immunocytochemical staining for the proapoptotic bcl-2 family member, bak, was performed. Frozen sections were stained with a monoclonal anti–human bak antibody (1 µg/mL) overnight at 4°C, and cytoplasmic staining was evaluated.

**Measurement of Neointima Formation**

In human veins, neointima formation was quantified from sections (n=6 per group) stained with Miller’s elastic van Gieson (EVG) and Mayer’s hematoxylin and eosin stains25 by use of image analysis (Image-Pro Plus version 3, Media Cybernetics). Neointimal area was measured in each of 3 pairs of serial sections, and the mean neointima thickness was determined by dividing by the length of the vein segment. The total number of neointimal cells 7 and 14 days after infection was counted and the mean calculated for each vein segment. The mean was divided by the length of the vein segment in millimeters to normalize for variations in the vein diameter between patients. Proliferation was assessed by continuous labeling with 10 µmol/L bromodeoxyuridine (BrdU) and immunocytochemical detection of positive nuclei as described.17 In pig vein grafts, 4 transverse sections at each of 3 equally spaced intervals were stained with EVG (n=6 grafts per group). For each section, the neointimal luminal surface, internal elastic lamina, and external elastic lamina were identified and traced from digital images, and neointimal and medial areas were calculated. Endothelial cell coverage was also quantified. For human saphenous veins, the number of cells positive for the endothelial cell marker QBend10 was counted (n=6 per group) and expressed per millimeter of vein length. In pig vein grafts, the percentage luminal coverage was calculated by image analysis after DBA lectin staining (n=4 per group).

**Statistical Analysis**

Analysis was performed by 2-way ANOVA for multiple comparisons between groups. Values were considered significant at P<0.05. Values are expressed as the mean±SEM.

**Results**

**Adenovirus-Mediated Gene Transfer to Porcine Saphenous Vein**

Adenovirus-mediated gene transfer to human saphenous vein has been described.18 We therefore initially characterized gene transfer to pig saphenous vein after graft placement in vivo. At 7 days after grafting, widespread β-galactosidase staining was observed throughout the surface of the graft, with no staining in the adjacent carotid artery (Figure 1). No staining was observed in vein grafts treated with vehicle alone. Quantification on cross sections revealed that 40±18% of surface cells were β-galactosidase–positive at 7 days (Figure 1; n=3), similar to the infection efficiency of human saphenous vein.18 However, analysis of grafts 28 days after infection demonstrated that only sporadic β-galactosidase–positive cells remained (data not shown).

**Recombinant TIMP-3 Localizes to the ECM**

Unlike TIMP-1 and -2, TIMP-3 is normally found within the ECM.26 Uninfected and RAdlacZ-infected human saphenous veins showed low levels of TIMP-3 located within the ECM of the media and neointima, and this was not increased with culture, consistent with previous observations (Figure 2A and 2C: RAd35-infected not shown).27 Infection with RAdTIMP-3 led to a substantial increase in TIMP-3 located within the ECM at the luminal surface and in the upper media (Figure 2B and 2D). Similar patterns were observed in pig vessels. TIMP-3 was undetectable in control carotid artery and in ungrafted saphenous vein (not shown). Low-level, diffuse TIMP-3 staining throughout the neointima, media, and adventitia was detected in vehicle- and RAdlacZ-treated control vein grafts at days 7 and 28 (Figure 2E and 2G). In contrast, high levels of TIMP-3 were observed in the ECM...
surrounding cells of RAdTIMP-3–treated vein grafts after 7 days (Figure 2F), which was still evident at 28 days after infection, albeit reduced (Figure 2H). Quantification of TIMP-3 overexpression by image analysis revealed that 26.5±7.0% of the luminal surface and upper media was immunoreactive for TIMP-3 (versus 2.2±0.7% for vehicle-treated and 0.7±0.2% for RAdlacZ-infected controls, \( P<0.05 \) versus both controls, \( n=3 \)), which remained elevated at 28 days after infection (11.2±2.5%). In contrast, TIMP-2 overexpression, like \( \beta \)-galactosidase in RAdlacZ-treated grafts, was evident at day 7 but not at day 28 (not shown).

**TIMP-2 or -3 Overexpression Potently Inhibits MMP Activity**

Elevated levels of pro-MMPs and activated gelatinases (MMP-2 and -9) are associated with the progression of vein graft neointima formation. Low-level MMP activity was detected in control uninfected human saphenous veins, consistent with previous observations,27 which increased with culture both in the media and in the neointima (Figure 3A and 3C). Overexpression of TIMP-3 substantially reduced gelatinase activity both at the luminal surface and in the medial layer (Figure 3B and 3D). MMP activity was greatly diminished even in regions with no detectable recombinant TIMP-3 immunoreactivity, ie, deeper medial layers. Zymography of tissue extracts revealed that TIMP-3 overexpression did not alter the absolute levels of MMP-2 or -9.

Low-level MMP activity was detected in ungrafted pig saphenous vein but was undetectable in control carotid arteries (not shown). Evaluation of vehicle- and RAdlacZ-treated veins revealed that MMP activity was high at day 7 after grafting both at the luminal surface and in the media (Figure 3E and 3F), and this was sustained at day 28 (not shown). Overexpression of TIMP-2 or TIMP-3 reduced MMP activity throughout the neointima and media 7 days after infection (Figure 3G and 3H) and was sustained at day 28 (not shown). Adventitial MMP activity was detectable, but low, in control vein grafts at days 7 and 28 and was unchanged by luminal overexpression of TIMP-2 or TIMP-3 (not shown).
Localized TIMP-3 but Not TIMP-2 Overexpression Promotes Apoptosis

To determine whether TIMP-3 overexpression promotes apoptosis, ISEL, TUNEL, and immunocytochemical staining for bak were performed. The numbers of ISEL-positive cells were low in control uninfected human veins in the neointima and in the media after 7 and 14 days (Figure 4A, Table 1). In contrast, in RAdTIMP-3-infected veins, both neointimal and medial cell apoptosis was significantly elevated from control values (Figure 4B, Table 1). Similar results were obtained with the TUNEL technique (Table 1) and by immunocytochemistry for cytoplasmic bak (Figure 4C and 4D, Table 1).

In pig vessels, no apoptosis was observed in control carotid artery or control ungrafted saphenous vein (not shown). ISEL showed that apoptosis in the neointima and media (combined score) was moderately induced in 7-day vein grafts (0.7±0.4% for vehicle-treated and 0.3±0.1% for RAdlacZ-treated, Figure 3E and 3F). Grafts infected with RAdTIMP-3 (4.5±1.1%) but not TIMP-2 (0.7±0.2%) showed significantly elevated levels of apoptosis (Figure 4; TIMP-2 not shown), P<0.05 versus both vehicle- and RAdlacZ-treated control groups, n=6). Colocalization studies revealed that 79±3% of ISEL-positive cells also stained for α-smooth muscle actin (Figure 4F, inset). Similar significant inductions in apoptosis were obtained with TUNEL (not shown) and immunocytochemistry for bak (Figure 4G and 4H). Apoptosis was undetectable in 28-day vein grafts (not shown).

Overexpression of TIMP-3 Inhibits Neointima Formation in Human and Pig Models

TIMP-3 significantly inhibited neointima formation at both days 7 (86%) and 14 (83%) after infection compared with uninfected controls (Figure 5, Table 2). Similarly, the number of neointimal cells per mm was significantly reduced in the RAdTIMP-3–treated group at both 7 and 14 days after infection, despite an increase in the neointimal proliferation index after 14 days (Table 2). No effect of TIMP-3 overexpression on medial cell density, medial proliferation rates, or endothelial cell coverage was observed (Table 2).
Control 28-day pig vein grafts (vehicle alone) had a consistent and pronounced neointima consisting mainly of α-actin–positive cells (Figure 6a). No effect on neointima formation was observed by infection with RAdlacZ (3.9±0.5 mm² for vehicle-treated and 4.8±1.1 mm² for RAdlacZ-treated vein grafts, n=6; Figure 6a, 6b, and 6e). In contrast, RAdTIMP-3 infection reduced neointima formation significantly, by 58% (2.0±0.1 mm², P<0.05, n=6 for Figure 3. TIMP-3 overexpression inhibits MMP activity. In situ zymography was used to assess MMP activity in human saphenous vein at day 7 (A and B) and day 14 (C and D) and in pig saphenous vein grafts at day 7 (E through H). Zones of MMP activity appear as white holes in dark background (which represent regions with no MMP activity or where MMP activity is inhibited). Uninfected control human saphenous veins (A and C) and vehicle-treated (E) and RAdlacZ-treated (F) pig vein grafts show high MMP activity, which is inhibited by TIMP-2 in pig (H) or TIMP-3 overexpression in human (B and D) and pig (G) tissues throughout vein. Arrows in A and C indicate neointimal/medial boundary and those in B, D, and E through H, luminal surface. Scale bar in A represents 50 μm and is applicable to A through D; in E it is 100 μm and applicable to panels E through H.

Figure 4. Effect of TIMP-3 overexpression on apoptosis in human saphenous vein (A through D) and pig interposition grafts (E through H). Apoptosis was evaluated by ISEL (A, B, E, and F) or bak immunostaining (C, D, G, and H) in control uninfected (A, C, E, and G) and RAdTIMP-3–infected (B, D, F, and H) human saphenous vein grafts at day 7 and in pig grafts at day 7. Insert in F demonstrates double staining for ISEL (red) and α-smooth muscle cell actin (brown) in a RAdTIMP-3–infected vein graft at day 7. Arrows in A, C, E, and G indicate neointimal/medial boundary. Arrowheads in B, D, F, and H indicate examples of positive nuclei for ISEL or positive immunostaining for bak. Scale bar in A represents 25 μm and is applicable to all panels.
RadTIMP-3–infected; Figure 6d and 6e) without affecting proliferation rates or endothelial cell coverage in day 7 or day 28 vein grafts (not shown). Surprisingly, when we investigated the effect on neointima formation of RadTIMP-2 infection in the pig model, it was significantly less than that of TIMP-3 (6.5±1.0 mm², P<0.05, n=6; Figure 6c and 6e). No effect of RadTIMP-3 on medial cross-sectional area was observed (7.1±1.2, 9.9±1.8, and 8.7±1.5 mm² for vehicle-, RadlacZ-, and RadTIMP-3–infected vein grafts, n=6; Figure 6f). Hence, intimal/medial ratio was also significantly reduced by TIMP-3 overexpression (Figure 6g).

### Table 1. Apoptosis Levels in Uninfected Control and RadTIMP-3–Infected Human Saphenous Vein Cultures

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Region of Vein</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>TIMP-3</td>
</tr>
<tr>
<td>ISEL</td>
<td>Neointima</td>
<td>2.3±0.9</td>
<td>16.4±4.2*</td>
</tr>
<tr>
<td></td>
<td>Media</td>
<td>5.8±1.3</td>
<td>21.0±3.9*</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Neointima</td>
<td>2.6±0.8</td>
<td>16.4±3.8*</td>
</tr>
<tr>
<td></td>
<td>Media</td>
<td>5.2±1.6</td>
<td>14.3±3.9*</td>
</tr>
<tr>
<td>bak</td>
<td>Neointima/media</td>
<td>6.1±0.7</td>
<td>27.4±9.9*</td>
</tr>
</tbody>
</table>

For bak analysis, positive cells from the neointima and media were pooled because of partial loss of morphology on frozen sections. All data are expressed as percentage positivity.

*P<0.05 for RadTIMP-3–infected vs uninfected control human saphenous veins.

### Discussion

In an attempt to inhibit the progression of neointima formation associated with late vein graft failure, we have focused on the ability of TIMP-3 to inhibit MMP activity and promote apoptosis when overexpressed. To accomplish this, we used adenoviral gene transfer in 2 established models of neointima formation, namely human saphenous vein organ cultures and pig saphenous vein–to–carotid artery interposition grafts. TIMP-3 was substantially overexpressed within the ECM at the luminal surface and in the upper media. MMP activity was inhibited...
throughout the veins, and apoptosis was induced, especially in regions of vein grafts with high TIMP-3 immunoreactivity. TIMP-3 overexpression significantly reduced neointima formation in human and pig models.

The immunolocalization of TIMP-3 is consistent with the ability of TIMP-3 to bind to the ECM. TIMP-3 secreted from locally infected cells therefore binds tightly to the ECM surrounding cells at the luminal surface and in the upper media, but not substantially within the ECM of distant sites. This pattern of expression may be advantageous in the context of inhibition of neointima formation as ECM localization induces high local concentrations of the inhibitor, even in the ECM surrounding noninfected cells.

In situ zymography demonstrated the pattern of gelatinase activity in pig interposition vein grafts and its inhibition by TIMP-2 or TIMP-3 overexpression. Control pig carotid arteries had no detectable MMP activity, in

| TABLE 2. Effect of TIMP-3 Overexpression on Neointima Formation in Human Saphenous Vein Cultures |
|-----------------------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                               | Day 4         | Day 7         | Day 14         |                  |                |
|                                               | Control       | TIMP-3        | Control        | TIMP-3          | Control        |
| Neointimal thickness, μm                      | 1.8±0.7       | 1.3±0.5       | 6.9±1.7        | 1.0±0.3*        | 18.8±4.9       |
| Neointimal cells/mm                           | 6.1±1.6       | 4.8±0.6       | 17.4±3.4       | 5.2±1.8*        | 28.4±3.9       |
| Medial density, cells/mm²                     | 1118±68       | 1277±133      | 1130±85        | 1237±184        | 1223±74        |
| Neointimal proliferation, %                   | 27.7±7.4      | 44.2±5.8      | 49±5.1         | 47±11.2         | 51.8±6.1       |
| Medial proliferation, %                       | 9.8±3.6       | 9.6±4.5       | 19.7±5.1       | 10.3±2.4        | 12.7±2.7       |
| Endothelial cells/mm                          | 9±3           | 9±2           | 5±1            | 5±1             | 5±1            |

*Statistical significance for RAdTIMP-3–infected veins vs uninfected controls (P<0.05).
agreement with findings in nondiseased human arteries. 28 Low-level MMP activity, however, was detected in control saphenous vein, and this substantially increased after grafting in all areas of the graft, analyzed at day 7, in agreement with results on cultured human veins. 27 MMP activity was still detected at day 28, albeit reduced and mainly restricted to the neointima. Overexpression of TIMP-2 or TIMP-3 inhibited MMP activity throughout both human veins in culture and pig veins in vivo, which demonstrates a substantial bystander effect, because only luminally exposed cells were transduced. TIMP-3 inhibition of MMPs in deeper regions of human and pig veins could occur if soluble TIMP-3 is produced after high-level overexpression, as occurs in isolated cultures. 21 Under these circumstances, ECM binding of TIMP-3 may become saturated by overexpression, resulting in the generation of local levels of a soluble form of TIMP-3. Alternatively, matrix-bound TIMP-3 may act as an immobilized sink for freely released MMPs.

In direct contrast to TIMP-3, TIMP-2 failed to induce apoptosis and inhibit neointima formation in pig vein grafts in vivo. We have previously reported that overexpression of TIMP-2 inhibits neointima formation in human saphenous veins solely through inhibition of MMP activity. 17 However, studies in the rat carotid artery balloon angioplasty model have demonstrated that adenovirus-mediated overexpression of TIMP-2 (or use of synthetic MMP inhibitors) blocked early migration of SMCs, although a “catch-up” effect prevented inhibition of neointima formation at later points. 19, 29 Our data here for TIMP-2 similarly indicate that the short-term overexpression of TIMP-2 achievable with first-generation adenoviruses is not sufficient to inhibit neointima formation in vivo. The ability of TIMP-3 to bind to the ECM prolongs its retention in the neointima even when adenovirus-mediated expression declines. This, together with the dual effect of MMP inhibition and induction of apoptosis by TIMP-3, allows effective inhibition of neointima formation in vein grafts in vivo.

Together, our data demonstrate that overexpression of TIMP-3 inhibits neointima formation in human saphenous veins in organ culture and in pig interposition grafts in vivo. The ability of TIMP-3 to inhibit MMPs and promote apoptosis, together with the bystander effect resulting from its secretion and binding to the ECM, makes TIMP-3 a highly attractive candidate for gene therapy for vein graft neointima formation.

Acknowledgments

This study was supported by the British Heart Foundation, the Medical Research Council for the UK, and the Garfield Western Foundation. We thank Jason Johnson, Catherine Keen, and Marcy Wyatt for technical assistance.

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Circulation. 2000;101:296-304
doi: 10.1161/01.CIR.101.3.296

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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