Diverse Contribution of Bone Marrow–Derived Cells to Vascular Remodeling Associated With Pulmonary Arterial Hypertension and Arterial Neointimal Formation

Makoto Sahara, MD; Masataka Sata, MD; Toshihiro Morita, MD; Kazuto Nakamura, MD; Yasunobu Hirata, MD; Ryozo Nagai, MD

Background—Recent evidence suggests that bone marrow (BM)–derived cells may differentiate into vascular cells that participate in arterial repair and/or lesion formation. However, it remains uncertain whether BM-derived cells also can participate in vascular remodeling associated with pulmonary arterial hypertension.

Methods and Results—The BM of Sprague-Dawley rats was reconstituted with that of green fluorescent protein–transgenic rats. The BM-chimeric rats were injected intraperitoneally with 60 mg/kg monocrotaline after unilateral subpneumonectomy, and they concurrently underwent wire-mediated endovascular injury in femoral artery. After 28 days, they had elevated right ventricular systolic pressure (58.8 ± 5.4 versus 20.4 ± 2.4 mm Hg in sham-control; *P* < 0.01). The pulmonary arterioles were markedly thickened, with an infiltration of green fluorescent protein–positive macrophages into the perivascular areas. The endothelium of pulmonary arterioles contained only a few green fluorescent protein–positive cells, and green fluorescent protein–positive cells were seldom detected as smooth muscle cells in the lesions of thickened pulmonary arterioles. In contrast, BM-derived smooth muscle–like cells could be readily detected in the thickened neointima and media of the wire-injured femoral artery. Moreover, intravenous injection of 1 × 10⁸ BM cells from young rats had no beneficial effects on pulmonary hypertension, pulmonary arterial remodeling, or survival in the aged rats treated with monocrotaline plus unilateral subpneumonectomy. No injected BM cell was identified as an endothelial cell or a smooth muscle cell.

Conclusions—These results suggest that BM-derived cells can participate in arterial neointimal formation after mechanical injury, whereas they do not contribute substantially to pulmonary arterial remodeling associated with monocrotaline-induced pulmonary arterial hypertension in the pneumonectomized rats. (Circulation. 2007;115:509-517.)

Key Words: bone marrow ■ hypertension, pulmonary ■ monocrotaline ■ myocytes, smooth muscle

Pulmonary arterial hypertension is a refractory disease characterized by a progressive increase in pulmonary artery pressure and resistance.¹–³ Although the origin of pulmonary arterial hypertension appears to be heterogeneous, it is generally accepted that the pulmonary vasculature initially undergoes persistent vasoconstriction and structural remodeling, leading to increased medial thickness of muscular arteries, peripheral extension of arterial muscularization, and increased matrix deposition.¹,³,⁴ This remodeling in pulmonary arterioles results in pulmonary hypertension, increased pulmonary vascular resistance, right ventricular hypertrophy, and right heart failure. While the pathogenesis of pulmonary arterial hypertension is poorly understood, it has been hypothesized that endothelial dysfunction or damage may trigger the pathogenesis of pulmonary arterial hypertension.⁵

Clinical Perspective p 517

Accumulating evidence suggests that bone marrow (BM)–derived cells may participate in the regeneration and remodeling of remote organs.⁶ Numerous studies have reported that circulating BM-derived endothelial progenitor cells (EPCs) play an important role in the repair of endothelial injury and in postnatal angiogenesis,⁷,⁸ suggesting that EPCs function to prevent pathological arterial remodeling. Furthermore, we and others have reported that BM-derived cells could potentially contribute to the pathogenesis of vascular diseases.⁹–₁³ It remains to be determined whether circulating BM-derived cells also participate in vascular remodeling of pulmonary arterioles during the development of pulmonary arterial hypertension, although a few studies suggest that transplantation of exogenous EPCs might hold therapeutic promise.¹⁴–₁₆
Here, we examined the potential participation of BM-derived cells in the pathogenesis of pulmonary arterial hypertension by injecting monocrotaline (MCT) after unilateral subpneumonectomy (USP) into the BM-chimeric rats.\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)

Furthermore, we rigorously tested and compared the contribution of BM-derived cells to vascular remodeling between pulmonary vasculature injured by MCT combined with USP and systemic arteries injured mechanically. Our results suggest little potential for BM-derived cells to participate in pulmonary arterial remodeling in a rat model of pulmonary arterial hypertension.

**Methods**

**Animals**

Wild-type Sprague-Dawley rats weighing 260 to 280 g were purchased from SLC (Shizuoka, Japan). Transgenic rats (Sprague-Dawley background) that ubiquitously express enhanced green fluorescent protein (GFP) were generous gifts from Dr. M. Okabe (Osaka University, Osaka, Japan).\(^1\)\(^9\)\(^10\) All procedures involving experimental animals were approved by the institutional committee for animal research at the University of Tokyo and complied with National Institute of Health guidelines.

**Induction of BM-Chimeric Rats**

Eight-week-old male Sprague-Dawley rats (n=30) were lethally irradiated with 14.0 to 15.0 Gy and injected intravenously with unfractionated BM cells (5×10\(^6\)) derived from 8-week-old male GFP-transgenic rats. Flow cytometric analysis revealed that peripheral blood cells of the recipient rats had been highly reconstituted with the injected donor cells (89.2±6.8%; range, 72.9% to 97.7%), as detailed in the online Data Supplement.

**Animal Models of Pulmonary Arterial Hypertension**

Four weeks after successful BM transplantation, the chimeric rats were classified into 3 groups (n=6 to 10 per group). In the MCT+USP group, the chimeric rats initially underwent right subpneumonectomy to resect the anterior and middle lobes of right lung via right intercostal thoracotomy. Seven days after right subpneumonectomy, the rats were injected intraperitoneally with 60 mg/kg MCT (Wako, Osaka, Japan). In the MCT or sham-control group, the chimeric rats were injected intraperitoneally with 60 mg/kg of MCT or saline alone, as described in the online Data Supplement.

**Measurement of Right Ventricular Systolic Pressure**

At 14 and 28 days after MCT injection, right ventricular systolic pressure (RVSP) of the rats was measured with polyethylene catheters. After RVSP was measured, the rats were euthanized, and the hearts and lungs were harvested. The ratio of the right ventricle to left ventricle plus septum weight (RV/LV ratio) was then determined. The lungs were fixed in a 4% paraformaldehyde and embedded in paraffin or plastic resin, as detailed in the online Data Supplement.

**Young BM Injection Into Nonirradiated Old Rats With Pulmonary Arterial Hypertension: The Nonirradiated Model**

To test the hypothesis that transplantation of BM cells from a young donor can inhibit pathological vascular remodeling associated with pulmonary hypertension,\(^2\)\(^0\)\(^2\)\(^1\) 6-month-old male Sprague-Dawley rats without irradiation were given 60 mg/kg MCT after right subpneumonectomy. Then, 1×10\(^6\), 1×10\(^7\), or 1×10\(^8\) unfractionated BM cells from 4- to 6-week-old male GFP-transgenic rats or PBS alone were intravenously injected into the old rats at 1, 7, 14, and 21 days after MCT injection. Each group had 15 rats. Ten rats were used for survival analysis; the rest were used for the measurement of RVSP and histological analysis of lungs.

**Wire-Mediated Endovascular Injury in BM-Chimeric Rats With Pulmonary Arterial Hypertension: The Simultaneous Injury Model**

To rigorously compare the contribution of circulating BM-derived cells to arterial remodeling after mechanical vascular injury with their contribution to pulmonary arterial remodeling associated with pulmonary hypertension, endovascular injury was induced in a separate series of the BM-chimeric rats (n=10). Seven days after right subpneumonectomy, the BM-chimeric rats underwent wire-mediated endovascular injury in the right femoral artery with an inserted guidewire (0.46-mm diameter). Then, 60 mg/kg MCT IP was administered to the rats on the same day. At 4 weeks, the lungs and femoral arteries were harvested for histological analysis.

**Histological Analysis**

Paraffin-embedded sections were processed for hematoxylin and eosin and subjected to elastic van Gieson and immunohistochemical stainings for examination by light microscopy. For analysis of the medial wall thickness of the pulmonary arterioles, the external diameter and medial wall thickness were measured in 30 muscular arteries (<200-μm external diameter) per lung section. The medial wall thickness was calculated as follows: percent wall thickness=[(medial thickness×2)/external diameter]×100.

**Immunohistochemistry**

The paraffin-embedded sections were incubated with primary antibodies (alkaline phosphatase–conjugated anti-α-smooth muscle actin [α-SMA], clone 1A4, Sigma, St Louis, Mo; anti-rat CD31 [PECAM-1], clone TLD-3A12, BD Biosciences, San Jose, Calif; and anti-CD68, clone ED1, Serotec, Oxford, UK), followed by incubation with biotinylated anti-mouse IgG secondary antibody (Dako, Glostrup, Denmark) and subsequent use of the avidin-biotin complex technique and Vector Red substrate (Vector Laboratories, Burlingame, Calif). The nuclei were counterstained with hematoxylin.

**Double-Immunofluorescence Study**

The plastic-embedded sections were incubated with primary antibodies (Cy3-conjugated anti-α-SMA, Sigma; anti-rat CD31, BD Biosciences; and anti-CD68, clone ED1, Serotec), followed by incubation with Cy3-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pa). The nuclei were counterstained with Hoechst 33258 (Sigma). The sections were observed under a confocal microscope (FLUOVIEW FV300, Olympus, Tokyo, Japan). α-SMA- or CD31-positive cells were counted in each pulmonary arteriole <200 μm in external diameter. At 28 days after MCT injection, 50 to 60 different pulmonary arterioles were analyzed in each rat.\(^9\)\(^11\)

**TUNEL Staining**

To detect apoptotic cell death, TUNEL staining with immunofluorescence double staining was performed in a separate experiment with wild-type Sprague-Dawley rats (n=12), as detailed in the online Data Supplement.

**Transmission Electron Microscopy**

An electron microscopic study was performed to identify apoptotic cells, as described in the online Data Supplement.

**Statistical Analysis**

Data are presented as mean±SD. Comparisons of means were evaluated by 1-way ANOVA, followed by Scheffé’s post hoc test. The Wilcoxon rank-sum test was performed for nonnormal continuous data. Survival curves were analyzed with the Kaplan-Meier method and compared through the Wilcoxon rank-sum test. Statistical significance was defined as \(P<0.05\).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
**Results**

**Pulmonary Hypertension and Pulmonary Arterial Remodeling Induced by MCT Combined With USP**

At 28 days after MCT injection, RVSP of the MCT group (50.1 ± 5.2 mm Hg; *P* < 0.01 versus sham-control) or MCT + USP group (58.8 ± 5.4 mm Hg; *P* < 0.01 versus sham-control) was significantly higher than that of sham-control group (20.4 ± 2.4 mm Hg) (Figure 1A). Consistently, the RV/LV ratio was significantly increased in the MCT group (0.45 ± 0.08; *P* < 0.01 versus sham-control) and MCT + USP group (0.48 ± 0.08; *P* < 0.01 versus sham-control) compared with the sham-control group (0.19 ± 0.02) (Figure 1B). The medial walls of the pulmonary arterioles were markedly thickened by MCT (Figure 1C), and percent wall thickness of the MCT group (60.9 ± 7.7%; *P* < 0.01 versus sham-control) and MCT + USP group (74.6 ± 8.6%; *P* < 0.01 versus sham-control) was significantly larger than that of sham-control group (22.3 ± 3.9%). The thickened media was composed exclusively of αSMA-positive cells (Figure 1D). Many macrophages infiltrated into the perivascular areas of pulmonary arterioles in the MCT-injured lung (Figure 1E). These pathophysiological and histological changes were similar to those observed in age-matched rats treated with MCT or MCT + USP without irradiation and BM transplantation (data not shown). MCT combined with USP induced more severe pulmonary hypertension and medial wall thickening of pulmonary arterioles compared with MCT alone (*P* < 0.05 in RVSP and percent wall thickness).

**Little Participation of BM-Derived Cells in the Pathological Remodeling of Pulmonary Arterioles**

The results of a double-immunofluorescence study in the lung tissue of BM-chimeric rats with or without pulmonary hypertension are shown as Figures 2 through 4. First, GFP signals were ubiquitously detected in the lung tissue of GFP-transgenic rats, including pulmonary arterioles composed of vascular smooth muscle cells and vascular endothelial cells, pulmonary bronchioles and alveoli, and hematocytes, as was expected (Figures 2A, 2D, and 3A). At 28 days after MCT injection, many BM-derived GFP-positive cells, which were considered hematocytes, were detected in the lung tissue of the BM-chimeric rats, but we seldom detected GFP-positive cells among αSMA-positive cells in the remodeled pulmonary arterioles (0.11 ± 0.12% in the MCT and MCT + USP groups) (Figure 2C, 2E, 2F and the Table). In addition, only a few GFP-positive cells expressed CD31 in the endothelium of the injured pulmonary arterioles (1.5 ± 0.7% in the MCT and MCT + USP groups) (Figure 3 and the Table), although the rate of the CD31/GFP double-positive cells among CD31-positive cells was ∼10-fold greater than the rate of the αSMA/GFP double-positive cells among αSMA-positive cells in the remodeled pulmonary arterioles. Many
of the GFP-positive cells were identified as macrophages in the perivascular areas, as determined by immunofluorescence staining against CD68 (Figure 4).

Effect of Young BM Injection on Pulmonary Hypertension Induced by MCT Combined With USP

Next, we investigated the therapeutic effects of exogenous young BM cells on pulmonary hypertension and on pulmonary arterial remodeling induced by MCT combined with USP. The old rats treated with MCT+USP received 1×10^6, 1×10^7, or 1×10^8 unfractionated BM cells from young GFP rats or PBS alone 4 times after MCT injection. In all 4 groups, RVSP and the RV/LV ratio were progressively increased in a similar manner (Figure 5A and 5B), and the BM transplantation had no effect on preventing the increase in RVSP or the RV/LV ratio. In addition, there was no improvement in survival resulting from BM transplantation (Figure 5C). At 28 days after MCT injection, the medial walls of the pulmonary arterioles were markedly thickened in all 4 groups (Figure 5D and 5E). We never detected GFP-positive cells derived from the injected cells in the pulmonary arterioles, although the injected GFP-positive cells were occasionally detected in lung tissue and peripheral blood (Figure 5E).

Thromboembolic Formation and Endothelial Cell Apoptosis in Pulmonary Arterioles and Capillaries After MCT Injection

We histologically characterized pulmonary arterioles (20 to 100 μm in external diameter) and capillaries in the lungs treated with MCT or MCT+USP. Thromboembolic formation, by which pulmonary arterioles were occluded, was readily detected after MCT injection. The number of the occluded pulmonary arterioles with thromboemboli was in-
increased in a time-dependent manner (Figure 6A and 6B). Similarly, TUNEL staining revealed that MCT induced apoptosis in a number of endothelial cells, but not medial smooth muscle cells, of the remodeled pulmonary arterioles with time (Figure 6C and 6D). A transmission electron microscopic study also was performed to identify cells with ultrastructural changes caused by apoptosis (Figure 6E). Endothelial cells of the capillaries occluded by thromboemboli displayed typical apoptotic morphology, including compaction of nuclear chromatin, nuclear fragmentation, and cytoplasmic condensation, in the lung at 28 days after MCT treatment.

**Figure 4.** Marked infiltration of BM-derived macrophages into the perivascular areas of the remodeled pulmonary arterioles injured by MCT or MCT+USP. The anti-CD68 (red) immunofluorescent staining was followed by counterstaining with Hoechst 33258 (blue) of pulmonary arterioles in the BM-chimeric rats. Yellow indicates the colocalization of GFP (green) and CD68, ie, BM-derived GFP-positive macrophages. A, The pulmonary arteriole of the BM-chimeric rat without pulmonary hypertension (sham-control). GFP-positive macrophages in the perivascular areas were rare, indicating no inflammatory responses there. B and C, Many GFP-positive macrophages (arrowheads) were infiltrated into the perivascular areas of the remodeled pulmonary arterioles in the MCT (B) or MCT+USP (C) groups, indicating severe inflammatory responses there. Scale bars, 100 μm (top) and 20 μm (bottom). Br indicates bronchiole; PA, pulmonary arteriole.

**Discussion**

In this study, we found that BM-derived cells did not participate substantially in pulmonary arterial remodeling associated with MCT-induced pulmonary hypertension in the pneumonectomized rats. Neither endogenous BM-derived progenitors nor systemically injected exogenous BM cells significantly contributed to pulmonary arterial remodeling, whereas BM-derived smooth muscle–like cells were readily detected in the femoral artery after wire-mediated endovascular injury was induced in the same rats. 

There is accumulating evidence that peripheral blood contains BM-derived progenitors of endothelium-like cells and/or smooth muscle–like cells, which may contribute to the process of arterial repair and remodeling. EPCs are supposed to be mobilized from BM into the peripheral blood in response to tissue ischemia or endothelial injury, to migrate to the sites of injury, and to differentiate into endothelium-like cells in situ. It is the generally accepted view that endothelial dysfunction or injury triggers the pathogenesis of pulmonary hypertension, including the obliteration of small arteries by thrombosis in situ, the infiltration of inflammatory cells, and the proliferation of vascular smooth muscle cells. These findings suggest that the administration of EPCs may have therapeutic effects on pulmonary hypertension and pulmonary arterial remodeling by accelerating endothelial healing of damaged pulmonary arterioles.

**Table 1.** Number of GFP-Positive Cells Among αSMA- or CD31-Positive Cells in Pulmonary Arterioles in Rats With Pulmonary Hypertension at 28 Days After MCT Injection

<table>
<thead>
<tr>
<th></th>
<th>Smooth Muscle Cells, GFP+ Cells/αSMA+ Cells</th>
<th>Endothelial Cells, GFP+ Cells/CD31+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MCT-28</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 1</td>
<td>1/5638</td>
<td>2/438</td>
</tr>
<tr>
<td>Rat 2</td>
<td>3/7290</td>
<td>3/516</td>
</tr>
<tr>
<td>Rat 3</td>
<td>2/6467</td>
<td>6/387</td>
</tr>
<tr>
<td>Rat 4</td>
<td>0/4891</td>
<td>7/469</td>
</tr>
<tr>
<td>Rat 5</td>
<td>10/8304</td>
<td>6/610</td>
</tr>
<tr>
<td>Rat 6</td>
<td>6/7718</td>
<td>14/546</td>
</tr>
<tr>
<td>Rat 7</td>
<td>5/5452</td>
<td>9/629</td>
</tr>
<tr>
<td><strong>MCT-28 + USP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat 1</td>
<td>4/6350</td>
<td>8/576</td>
</tr>
<tr>
<td>Rat 2</td>
<td>23/5118</td>
<td>11/484</td>
</tr>
<tr>
<td>Rat 3</td>
<td>8/5373</td>
<td>4/430</td>
</tr>
<tr>
<td>Rat 4</td>
<td>7/7097</td>
<td>15/515</td>
</tr>
<tr>
<td>Rat 5</td>
<td>3/6784</td>
<td>7/596</td>
</tr>
<tr>
<td>Rat 6</td>
<td>14/5728</td>
<td>6/478</td>
</tr>
</tbody>
</table>

MCT-28 and MCT-28 + USP indicate rats in the MCT group (MCT alone) and MCT+USP group at 28 days after MCT injection, respectively. The GFP-positive cells among αSMA- and CD31-positive cells were counted in 50 to 60 different pulmonary arterioles (≤200-μm external diameter) under a confocal microscope.
arterioles. Injection of EPCs has been demonstrated to restore microvascular structure and function in rats and dogs. Transplantation of genetically modified EPCs improved pulmonary hypertension and survival in rats treated with MCT. In these studies, the transplanted EPCs were successfully incorporated into the pulmonary endothelium. In contrast, we found that untreated total BM-derived cells were relatively rarely incorporated into the MCT-injured pulmonary endothelium, although the contribution of BM cells to endothelium was 10-fold greater than that to the medial layers in the remodeled pulmonary arterioles. The different sources of the transplanted cells (ie, circulating BM-derived cells, isolated unfractionated BM cells, or ex vivo expanded EPCs) may account for this discrepancy. Indeed, the incorporation rate of ex vivo expanded EPCs to newly formed vessels (7% to 20%) appears to be higher than that achieved by mobilization of endogenous BM cells (<2%). Moreover, it has been reported that transplantation of adrenomedullin- or endothelial nitric oxide synthase–transduced EPCs causes significantly greater improvement in pulmonary hypertension and survival than transplantation of untreated EPCs. Thus, it would be a relatively rare occurrence for endogenous untreated BM-derived progenitor cells to be incorporated into pulmonary vasculature in rats treated with MCT combined with USP.

We also found that BM-derived cells seldom contribute to medial thickening associated with MCT-induced pulmonary hypertension in the pneumonectomized rats. In contrast, Davie et al have reported that circulating BM-derived “progenitor” cells, defined as c-kit–positive cells, were involved in vessel wall thickening of pulmonary arterioles through infiltration into the adventitia via the vasa vasorum in a bovine model of hypoxia-induced pulmonary hypertension. It remains to be determined whether those c-kit–positive cells infiltrating into the adventitia differentiate into smooth muscle–like cells that contribute to medial thickening. It is plausible that most of the BM-derived cells represent macrophage or myeloid lineage cells that were recruited by inflammatory signals. In fact, we observed that MCT treatment led to increased accumulation of BM-derived cells around pulmonary arterioles as adventitial inflammatory cells. It is likely that these adventitial inflammatory cells secrete various cytokines that may play a role in the pathogenesis of pulmonary hypertension.

We and others have previously reported that BM can give rise to vascular cells that participate not only in repair but also in lesion formation. These progenitor cells are considered smooth muscle–like progenitor cells. However, the present study has revealed that BM-derived cells seldom transdifferentiate into vascular smooth muscle cells that contribute to pathological medial thickening of the MCT-injured pulmonary arterioles, whereas BM-derived cells significantly contributed to vascular remodeling after wire-mediated endovascular injury in the same rats. We previously reported that...
the contribution of BM cells to arterial remodeling in mice depends heavily on the type of injury. When the artery was severely injured, BM-derived cells contributed significantly to lesion formation and differentiated into smooth muscle–like cells. MCT-injured pulmonary arterioles and capillaries were frequently occluded by thrombosis in situ with endothelial cell apoptosis. Thrombosis in situ often is found in small pulmonary arterioles in patients with pulmonary arterial hypertension. It is likely that a series of thromboses in peripheral pulmonary circulation would cause increased pulmonary vascular resistance and impose excess pressure on pulmonary arterioles proximal to vascular obstructive lesions. The medial layer could be thickened by an increase in muscle mass in response to the elevated pulmonary arterial pressure, even in the absence of direct vascular injury. In contrast, a wire-mediated endovascular injury causes endothelial denudation and mechanical dilatation of the vessel with massive apoptosis of medial smooth muscle cells, whereas the present study revealed that seldom or never was there apoptosis of medial smooth muscle cells in the remodeled pulmonary arterioles as determined by TUNEL staining. Accordingly, the apparent difference in the process of vascular remodeling between pulmonary and femoral vasculature might account for the diverse contribution of BM-derived cells to lesion formations.

Conclusions
The present study suggests that circulating BM-derived cells can contribute to neointimal formation after mechanical injury, whereas they do not contribute substantially to pulmonary arterial remodeling associated with MCT-induced pulmonary hypertension in the pneumonectomized rats. The difference in the pathogenesis of occlusive vascular remodeling seems to account for the diverse contribution of BM-derived cells to vascular remodeling associated with pulmonary arteriole thickening in pulmonary arterial hypertension and neointimal hyperplasia after arterial injury.

Sources of Funding
This study was supported in part by grants from Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare.
Disclosures

None.

References


Diverse Contribution of Bone Marrow–Derived Cells to Vascular Remodeling Associated With Pulmonary Arterial Hypertension and Arterial Neointimal Formation
Makoto Sahara, Masataka Sata, Toshihiro Morita, Kazuto Nakamura, Yasunobu Hirata and Ryozo Nagai

_Circulation_. 2007;115:509-517; originally published online January 22, 2007;
doi: 10.1161/CIRCULATIONAHA.106.655837
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 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/115/4/509

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2007/01/26/CIRCULATIONAHA.106.655837.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/