Adenoviral Human BCL-2 Transgene Expression Attenuates Early Donor Cell Death After Cardiomyoblast Transplantation Into Ischemic Rat Hearts

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Background—Cell transplantation for myocardial repair is limited by early cell death. Gene therapy with human Bcl-2 (hBcl-2) has been shown to attenuate apoptosis in the experimental setting. Therefore, we studied the potential benefit of hBcl-2 transgene expression on the survival of cardiomyoblast grafts in ischemic rat hearts.

Methods and Results—H9c2 rat cardiomyoblasts were genetically modified to express both firefly luciferase and green fluorescent protein (mH9c2). The cells were then transduced with adenovirus carrying hBcl-2 (AdCMVhBcl-2/mH9c2). Lewis rats underwent ligation of the left anterior descending artery (LAD) to induce a sizable left ventricular (LV) infarct. Hearts were explanted and the infarcted region was restored using collagen matrix (CM) seeded with 1×10^6 mH9c2 cells (n=9) or AdCMVhBcl-2/mH9c2 cells (n=9). Control animals received CM alone (n=6) or no infarct (n=6). Restored hearts were transplanted into the abdomen of syngeneic recipients in a “working heart” model. Cell survival was evaluated using optical bioluminescence imaging on days 1, 5, 8, 14, and 28 after surgery. The left heart function was assessed 4 weeks postoperatively using echocardiography and magnetic resonance imaging. During 4 weeks after surgery, the optical imaging signal for the AdCMVhBCL2/mH9c2 group was significantly (P<0.05) higher than that of the mH9c2-control group. Both grafts led to better fractional shortening (AdCMVhBcl-2/mH9c2: 0.21±0.03; mH9c2: 0.21±0.04; control: 0.15±0.03; P=0.04) and ejection fraction (AdCMVhBcl-2/mH9c2: 47.0±6.2; mH9c2: 48.7±6.1; control: 34.3±6.0; P=0.02) compared with controls. Importantly, no malignant cells were found in postmortem histology.

Conclusion—Transduction of mH9c2 cardiomyoblasts with AdCMVhBcl-2 increased graft survival in ischemic rat myocardium without causing malignancies. Both AdCMVhBcl-2/mH9c2 and mH9c2 grafts improved LV function. (Circulation. 2006;114[suppl I]:I-174–I-180.)

Key Words: apoptosis ■ cells ■ gene therapy ■ grafting ■ myocardial infarction

Stem cell therapy is a promising therapeutic strategy for treating ischemic heart failure.1–3 The viability of cell grafts is affected by several adverse environmental factors of the injured ischemic host myocardium.4 Inflammatory host response leading to oxidative stress can readily induce graft apoptosis,5,6 markedly reducing the number of surviving donor cells. Early graft death is a major unsolved problem in stem cell therapy and demands supportive therapeutic approaches, such as growth factor administration or gene therapy.7,8

Apoptosis is regulated by a balance of various genes either promoting or inhibiting programmed cell death.9,10 Overexpression of the anti-apoptotic Bcl-2 gene has been found in cardiac fibroblasts, which are well-known to be resistant to most situations that compromise cell survival.10 Recent studies have shown that after acute myocardial infarction9 or during cardiac posttransplant ischemia/reperfusion injury,11 Bcl-2 expression is significantly induced to limit uncontrolled cell death. We have also shown previously that intracoronary injection of an adenovirus carrying the human Bcl-2 gene limits both apoptosis and free radical release in a rat heart transplant model.6 The Bcl-2 gene is known not only for regulating apoptosis but also controls nonapoptotic cell death depending on autophagy genes.12 Recently, transgenic embryonic stem cell lines expressing Bcl-2 were found to self renew continuously, even under serum-free and feeder cell-free conditions.8

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Presented at the American Heart Association Scientific Sessions, Dallas, Tex, November 13–16, 2005.

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

DOI: 10.1161/CIRCULATIONAHA.105.001370

I-174
In the present study, we tested the hypothesis that cardiomyoblast grafts expressing human Bcl-2 may be more resistant to apoptosis and thus may lead to enhanced graft survival in ischemic rat myocardium. First, for intra-individual comparison of cell grafts, we developed a new model of cell transplantation into the abdominal wall. Second, using a rat heterotopic working heart transplant model, we restored a major portion of the ischemic left ventricle by implanting collagen matrix seeded with H9c2 rat embryonic cardiomyoblasts. H9c2 cells stably expressing firefly luciferase and green fluorescent protein (mH9c2) were transduced with adenovirus carrying the hBcl2 gene (AdCMVhBcl2-2/mH9c2). These cells were compared with mH9c2 controls in terms of in vivo cell survival courses using optical bioluminescence. Resulting left heart function was assessed by echocardiography and magnetic resonance imaging (MRI).

Methods

Animal Care
All surgical interventions and animal care were provided in accordance with The Guide for the Care and Use of Laboratory Animals (National Institutes of Health, volume 25, no 28, revised 1996).

Lentiviral Vector-Mediated Cell Labeling and Adenoviral hHBcl2 Transduction
H9c2 rat cardiomyoblasts (American Type Culture Collection) were cultured and maintained as previously described. Cells were genetically labeled by exposing them to 1 × 10⁷ pfu of lentivirus carrying a cytomegalovirus (CMV) promoter driving the expression of a firefly luciferase reporter gene (fluc) and a green fluorescent protein (GFP) gene separated by an internal ribosomal entry site element. The genetically modified cells (mH9c2) then underwent two rounds of FACS sorting followed by a single clone selection. The brightest clone, as confirmed by in vitro firefly luciferase assay, was used for further experiments.

For hBcl-2 transduction of H9c2 cells a recombinant, E1 deleted adenovirus carrying the human Bcl-2 gene (AdCMVhBcl2-2) was used (David T. Curiel, MD, University of Alabama, Birmingham, Birmingham, Ala) as described before. An empty vector, Ad5BglII (AdvNull) (University of Iowa Gene Therapy Center, Birmingham, Ala) as described before. The multiplicity of infection (MOI) was 100, 200, and 400 (n = 9) cells or 1 × 10⁷ AdvNull or mH9c2 (n = 6/group), for AdvNull a MOI of 200 was used. Cells were incubated at 37°C to 70 minutes and the average values did not differ between groups. After 24 hours cells were transduced with AdCMVhBcl2-2, AdvNull, or media alone. For AdCMVhBcl2, the multiplicity of infection (MOI) was 100, 200, and 400 (n = 6/group), for AdvNull a MOI of 200 was used. Cells were incubated at 37°C (5% CO₂) for 1 hour, then washed and recultured, with the medium replenished in 24-hour intervals. At 72 hours after adenoviral infection, cells were collected by trypsinization.

After adjustment of protein content, an enzyme-linked immunosorbent assay (ELISA) Kit was used to confirm successful hBcl2 transduction (Oncogene, San Diego, Calif). Light absorbance was read at 450 nm wavelength and data were displayed as units per milligram (U/mg) of total protein (Figure 1).

Cell Transplantation Into the Cryoinjured Abdominal Wall
Five male Lewis rats (240 to 280 grams, Harlan Sprague-Dawley) were anesthetized as described. The animals were intubated, mechanically ventilated, and a sternotomy was performed. To induce a sizable left ventricular myocardial infarct, the proximal LAD was ligated. Successful infarction was determined by observing a pale discoloration of the infarcted area. Five minutes later, the ascending aorta was clamped and the heart was perfused with cold cardioplegic solution. The donor heart was explanted and placed on cold saline for ex situ myocardial restoration. An end-to-end anastomosis of the left atrium to the pulmonary artery was performed. The anterior portion of the left ventricle was dissected creating a 4 × 4-mm semi-lunar pouch within the infarcted area. After intramyocardial implantation of a 3 × 3 × 1-mm moist block of sterile Gelfoam (GF); purified pork skin gelatin; Upjohn), the hearts were transplanted into the abdomen of syngeneic recipients performing an aorto-aortic anastomosis and an anastomosis of the donor superior vena cava to the recipient inferior vena cava. The ischemia time for the hearts ranged from 60 to 70 minutes and the average values did not differ between groups.

Before reperfusion, 1 × 10⁶ mH9c2 (n = 9) cells or AdvNull/mH9c2 (n = 9) cells suspended in 50 μL liquid, pre-chilled growth factor-reduced Matrigel (MG) were injected into the reconstructed area. Six infarcted hearts with plain GF implantation and 6 hearts without infarct were transplanted as controls (Table 1).

Before wound closure, an intraperitoneal catheter system with a subcutaneous injection site was implanted to allow error-free substrate delivery for bioluminescence imaging. To avoid cell rejection (allogenic H9c2 cells) all animals received cyclosporine (7.5 mg/kg per day orally).

In Vivo Optical Bioluminescence Imaging
We used optical bioimaging (IVIS 50, Xenogen) for in vivo detection of genetically labeled cells. Rats were placed in the imaging chamber under continuous isoflurane inhalational anesthesia. The animals were rotated to the left to expose the abdominal heterotopic heart to the Charge-Coupled Device (CCD) camera. After acquisition of a baseline image, rats were injected with D-Luciferin, 400 mg/kg body weight intraperitoneally via the port catheter system. Rats were imaged on day 1, 5, 8, 14, and 28 after surgery using 5-minute acquisition scans until peak levels were reached. For the abdominal wall model the animals were imaged on growth factor reduced Matrigel (MG) (Becton Dickinson Labware). The lower sites were transplanted with 0.5 × 10⁷ normal mH9c2 cells diluted in MG. Cell survival was evaluated longitudinally using optical bioluminescence imaging on days 1, 5, and 10 after surgery.

Myocardial Restoration and Heterotopic Heart Transplantation
Male Lewis rats (240 to 280 grams, Harlan Sprague-Dawley) were anesthetized as described. The animals were intubated, mechanically ventilated, and a sternotomy was performed. To induce a sizable left ventricular myocardial infarct, the proximal LAD was ligated. Successful infarction was determined by observing a pale discoloration of the infarcted area. Five minutes later, the ascending aorta was clamped and the heart was perfused with cold cardioplegic solution. The donor heart was explanted and placed on cold saline for ex situ myocardial restoration. An end-to-end anastomosis of the left atrium to the pulmonary artery was performed. The anterior portion of the left ventricle was dissected creating a 4 × 4-mm semi-lunar pouch within the infarcted area. After intramyocardial implantation of a 3 × 3 × 1-mm moist block of sterile Gelfoam (GF); purified pork skin gelatin; Upjohn), the hearts were transplanted into the abdomen of syngeneic recipients performing an aorto-aortic anastomosis and an anastomosis of the donor superior vena cava to the recipient inferior vena cava. The ischemia time for the hearts ranged from 60 to 70 minutes and the average values did not differ between groups.

Before reperfusion, 1 × 10⁶ mH9c2 (n = 9) cells or 1 × 10⁷ AdCMVhBcl2-2/mH9c2 (n = 9) cells suspended in 50 μL liquid, pre-chilled growth factor-reduced MG were injected into the reconstructed area. Six infarcted hearts with plain GF implantation and 6 hearts without infarct were transplanted as controls (Table 1).

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days 1, 5, and 10 after surgery. Peak signal in terms of total flux (photons/sec) from a fixed region of interest (ROI) was evaluated using the Living Image 2.50 software (Xenogen).

**MRI and Echocardiography**

Heart function was evaluated using echocardiography (n/H11005 9/group) and MRI (n/H11005 3/group) 4 weeks after surgery. MRI (4.7 Tesla) was performed using a Unity Inova console (Varian, Inc) and a 15-cm horizontal bore magnet (Oxford Instruments, Ltd,) with GE Tefron Gradients (12 G/cm). For ECG gating, 2 subcutaneous precordial leads (SA Instruments, Inc) were used. LV function was evaluated using an ECG-triggered cine sequence (TE 2.8-ms, TR 160-ms, FA 60°, FOV 3.0 cm², matrix 128x128, slice gap 0-mm, slice thickness 1.0-mm, 8 NEX, and 12 cardiac phases). The data were analyzed using the MR Vision software (Winchester). LV ejection fraction (LVEF) was calculated by tracing the endocardial and epicardial borders in end-systole and end-diastole. Measurements were performed by an independent, blinded investigator (T.A.).

The Acuson Sequoia C256 echocardiography system (15.8 MHz) was used to obtain standardized parasternal long-axis and short axis views. M-mode images at the papillary muscle level were used to calculate fractional shortening. Examinations were evaluated by an independent experienced investigator (R.S.).

**Histology and Immunohistochemistry**

AdCMVhBcl-2/mH9c2 cells were trypsinized, resuspended in culture medium, centrifuged at 1000 rpm for 5 minutes, and fixed in 10% paraformaldehyde. Cells were then centrifuged and resuspended in phosphate-buffered saline (PBS), and, finally, in dH2O before smearing on the slide. For staining, a rabbit anti-hBcl-2-antibody (Rockland) and a biotinylated donkey anti-rabbit IgG (Biomeda) were used.

At day 10 (abdominal wall experiments) and day 28 (cardiac restoration model) after surgery, hearts were excised, fixed in 2% paraformaldehyde for 2 hours, and cryoprotected in 30% sucrose overnight. Frozen tissue sections (6 μm, 4 levels per sample) were stained using the following primary antibodies: rabbit anti-sarcomeric-actin (Sigma-Aldrich), rabbit anti-connexin 43 (Sigma-Aldrich), and polyclonal Alexa Fluor 488-conjugated anti-GFP (Molecular Probes). Alexa 546-conjugated or Alexa 647-conjugated secondary antibodies were used (Molecular Probes). Nuclei were stained with Hoechst (Sigma-Aldrich). To identify tumor formation by AdCMVhBcl-2 transduced cells we performed hematoxylin and eosin (HE) and trichrome stains.

Infarct size was determined by computer-assisted planimetry (AutoCAD; Autodesk) and defined as the infarcted area divided by the total left ventricular area. Values were averaged over 4 level sections per sample.

**Statistics**

Data were expressed as mean±standard deviation (ELISA, MRI, echocardiography, histology) or mean±standard error of mean (Bioimaging). Data for heart function were compared using the Mann-Whitney U test. Bioimaging and ELISA data were compared using the Student t test (SPSS 11.0 for Windows). Differences were considered significant at P≤0.05.

**Authorship Responsibility**

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

Expression of Human Bcl-2 in Rat H9c2 Cardiomyoblasts

Human Bcl-2 was expressed in AdCMVhBcl-2/mH9c2 cardiomyoblasts as shown by anti-hBcl-2 immunohistochemistry. Cells stained brown after incubation with streptavidin-horseradish peroxidase and exposure to DAB. Staining was negative for plain mH9c2 cells and AdvNull/mH9c2 controls. The hBcl-2 ELISA revealed significantly greater human hBcl-2 protein content in AdCMVhBcl-2/mH9c2 cells than in either AdvNull/mH9c2 controls or plain mH9c2 cells (P<0.01 for all MOI of AdCMVhBcl-2 tested; comparisons made against either AdvNull/mH9c2 or mH9c2 cells). Adenoviral transduction of hBcl-2 was most effective with an MOI of 400 (Figure 1), which was used for further experiments.

Optical Bioluminescence: Cell Grafting Into the Abdominal Wall

Bioluminescence signals from hBcl-2 transduced cell grafts were markedly higher compared with mH9c2 controls throughout 10 days of in vivo observation (Figure 3). Only AdCMVhBcl-2/mH9c2 grafts were detectable in vivo at day 10, whereas signals from mH9c2 controls decreased to background level (<60 photons/sec×10^3). At day 5, bioluminescence signals obtained from injured regions were higher in both groups compared with signals from healthy control regions (P=0.06 for hBcl-2 and P<0.01 for controls; Figures 2, 3).

Optical Bioluminescence: Myocardial Restoration

In vivo bioluminescence signals were significantly higher in AdCMVhBcl-2/mH9c2 cells at days 1, 5, and 8 after surgery compared with mH9c2 controls (P<0.05). Whereas the signals of the control group decreased continuously during 4 weeks of monitoring, we found an increase of the bioluminescence signal in AdCMVhBcl-2/mH9c2 cells between day 1 and day 5 (Figure 4, Table 2). Although viable cells of both groups were detectable in vivo after 4 weeks, all signals decreased close to background level at day 28 (Table 2).

Histology and Immunohistochemistry

The histological screening of level sections (4 levels/sample, HE staining) performed by an experienced pathologist (A.C.) revealed no tumors or malignant cells in AdCMVhBcl-2/mH9c2 samples. Some of the transplanted mH9c2 cells assumed a spindle shape when they were in proximity with peri-infarct myocardium, indicating potential mechanical interactions between these cells and the host microenvironment (Figure 5A through 5C). However, when the cells were located far from the host myocardium (eg, in the center of the collagen matrix), they assumed a round shape (Figure 5G through 5I). The mH9c2 cells remained mononuclear and stained for α-sarcomeric actin, but not for connexin 43. Infarct sizes did not differ between groups (controls: 37±3%, AdCMVhBcl-2/mH9c2: 38±6%, mH9c2: 39±7%, P=not significant).

Echocardiography and MRI

Compared with heterotopic healthy control hearts (FS: 0.29±0.09; LVEF: 57±1%), transplantation of infarcted control hearts (GF without cells) led to significantly lower FS (0.15±0.04, P<0.01) and LVEF (34±6%, P<0.05).

Transplantation of mH9c2 cardiomyoblasts resulted in significantly better FS (mH9c2: 0.21±0.03; P=0.02) and LVEF (mH9c2: 48±8; P<0.05) compared with infarcted control hearts. There was no difference between AdCMVhBcl-2/mH9c2 grafts (FS: 0.21±0.04; P=0.02, LVEF: 47±6%; P<0.05) and normal mH9c2 grafts (Figure 6).

Discussion

As a proof of principle, the present study demonstrates that adenoviral hBcl-2 transduction of rat cardiomyoblasts is feasible and enhances early cell survival after transplantation. Overexpression of hBcl-2 in mH9c2 cells led to greater bioluminescence signals than nontransduced mH9c2 cells for...
the first 8 days after implantation into ischemic rat hearts. Notably, while the bioluminescence signals for the control cardiomyoblasts decreased to 70% of the baseline level within the first 5 days, the AdCMVbcl-2/mH9c2 cells showed an almost 75% increase in imaging signal between day 1 and day 5. These data strongly suggest that hBcl-2 expression may help to protect cells against apoptotic injury.5,6,9 Interestingly, the bioluminescence signals for the AdCMVbcl-2/mH9c2 cells decreased rapidly after the first week to background levels at day 28. This might be caused by the downregulation of hBcl-2 transgene expression as a result of inflammatory cytokines silencing the CMV promoter.17 It is possible that the AdCMVbcl-2/mH9c2 cells had lost their survival advantage over the mH9c2 cells by 4 weeks. Future

Figure 4. Optical bioluminescence imaging 1 and 5 days after cardiac cell grafting in a heterotopic heart transplant model. AdCMVbcl-2/mH9c2 cells showed significantly higher signals compared with mH9c2 controls.

### TABLE 2. In Vivo Bioluminescence Imaging

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<td>7456±2881#</td>
<td>1595±577+</td>
<td>338±77</td>
<td>201±17</td>
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<tr>
<td>MH9c2</td>
<td>1009±128*</td>
<td>705±75#</td>
<td>512±75†</td>
<td>324±56</td>
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Significantly increased imaging signal (photons/sec $\times 10^{3}$) for AdCMVbcl-2/mH9c2 cell grafts compared to mH9c2 controls at days 1 (*P=0.01), 5 (#P=0.03), and 8 (+P=0.05). The differences at day 28 may be caused by variability at low bioluminescence signal intensities.
studies using nonviral promoters (eg, ubiquitin, beta-actin) or less immunogenic virus for Bcl-2 delivery (eg, gutless adenovirus, lentivirus) will help to resolve the issue of transgene silencing, which may lead to longer hBcl-2 expression.

The early survival advantages of AdCMVhBcl-2/mH9c2 cells were independent of transplantation sites and were found also after transplantation into the abdominal wall muscle. AdCMVhBcl-2/mH9c2 cells showed greater bioluminescence signals at days 5 and 10, compared with control cells, after implantation into both cryoinjured and healthy host muscles. At day 5 cryoinjury of the host muscle appeared to have some beneficial effect on the survival of both AdCMVhBcl-2/mH9c2 and plain mH9c2 cells, compared with uninjured controls. The reason for this finding is not entirely clear and may be related to a differential rate of cell proliferation in the presence of muscle injury. Future histological studies using cell proliferation markers (eg, BrdU and Ki67) will help to elucidate the mechanism(s) by which muscle injury alters the survival kinetics of transplanted cells.

A major concern for this study was that hBcl-2 gene expression might induce tumor formation. Our histological analysis revealed cell clusters of round GFP-positive myoblasts. These cells were mononuclear, suggesting that they retained proliferative capacity and have not differentiated into multi-nucleated myotubes. No malignant cells or tumors were found. This is in contrast to our unpublished results showing

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**Figure 5.** AdCMVhBcl-2/mH9c2 cells expressing GFP (green) 4 weeks after cell transplantation. A to C, Few GFP-positive cells scattered throughout the host myocardium. D to F, Three GFP-positive cells expressing α-sarcomeric actin (red). G to I, A cluster of round mononuclear GFP-positive cells in an infarcted area. Nuclei are stained with Hoechst (blue).

**Figure 6.** Echocardiography and MRI 4 weeks after heterotopic working heart transplantation. Fractional shortening (FS) and left ventricular ejection fraction (LVEF) were significantly higher in mH9c2 grafted hearts compared with controls. No difference between hBcl-2 expressing cells and normal mH9c2 cells. *P<0.02, +P<0.02. #P<0.05. ^P<0.05.
teratoma formation after hBcl-2 transduction of mouse embryonic stem cells. Although increased expression of hBcl-2 appears to predispose some cell types to neoplasia, a recent study suggests that isolated overexpression of hBcl-2 is not oncogenic. Compared with pluripotent stem cells, differentiated cardiomyoblasts might be less susceptible to gene mutations.

As shown by our echocardiography and MRI data, mH9c2 tissue grafting improved left heart function. This finding is consistent with other experimental studies, which reported improved left heart function after cardiac cell grafting. Our mH9c2 cells did not express connexin 43, indicating that they do not electromechanically couple with host cardiomyocytes to actively contribute to contractile function. We believe that the collagen-based mH9c2 tissue grafts improved LV function via paracrine effects by influencing both angiogenesis and scar formation.

In this study, we used a unique combination of 2 innovative experimental models and reporter gene labeling technique for in vivo survival analysis of cellular grafts. The stable lentiviral luciferase/GFP transduction of the cardiomyoblasts provided reliable in vivo bioluminescence imaging and correlating histological cell identification. The newly developed abdominal wall model was designed for intra-individual comparison of cell grafts to exclude inter-individual variability in terms of host inflammation and D- Luciferin metabolism. The “working heart” heterotopic transplant model provided standardized myocardial infarction and controlled large-scale ex situ myocardial restoration. Importantly, compared with the standard heterotopic transplant technique it allowed for assessment of heart function.

In conclusion the present study demonstrates the feasibility of using anti-apoptotic hBcl-2 gene transduction for creation of robust embryonic cardiomyoblast cell grafts. As assessed with optical bioluminescence, hBcl-2 expression resulted in markedly improved cell survival during the first 5 days after transplantation. Left heart function improved after mH9c2 tissue grafting independent from hBcl-2 transduction.

Acknowledgments
We thank Pauline Chu for tissue processing and staining, Ramachandra Sista for assistance with echocardiography, and V. Mariano for animal care.

Sources of Funding
I.K. was supported by the annual Bayer Research Grant of the German Society of Cardiology (2004) and the annual Thoracic Research Scholarship (2004) of the German Society for Thoracic and Cardiovascular Surgery. G.v.D. was supported by a German Research Society grant (DE 740/1-1). This work was supported by the Falk Cardiovascular Research Fund (R.C.R.), NHLBI 5R01HL078632 (S.S.G.), NCF ICMIC P50 CA114747 (S.S.G.) and NCI SAIRP.

Disclosures
None.

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Circulation. 2006;114:I-174-I-180
doi: 10.1161/CIRCULATIONAHA.105.001370
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

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/114/1_suppl/I-174

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