Gene Expression Profiles and B-Type Natriuretic Peptide Elevation in Heart Transplantation
More Than a Hemodynamic Marker

Mandeep R. Mehra, MD; Patricia A. Uber, Pharm D; Dirk Walther, PhD; Mark Vesely, MD; Jay G. Wohlgemuth, MD; James Prentice, MS; Darren Tayama, MD; Margaret Billingham, MD

Background—B-type natriuretic peptide (BNP) is chronically elevated in heart transplantation and reflects diastolic dysfunction, cardiac allograft vasculopathy, and poor late outcome. This investigation studied peripheral gene expression signatures of elevated BNP concentrations in clinically quiescent heart transplant recipients in an effort to elucidate molecular correlates beyond hemodynamic perturbations.

Methods and Results—We performed gene microarray analysis in peripheral blood mononuclear cells of 28 heart transplant recipients with clinical quiescence (absence of dyspnea or fatigue; normal left ventricular ejection fraction [EF >55%]; ISHLT biopsy score 0 or 1A; and normal hemodynamics [RAP <7 mm Hg, PCWP ≤15 mm Hg, and CI ≥2.5 L/min per m²]). BNP levels were performed using the Triage B-type Natriuretic Peptide test (Biosite Diagnostics Inc, San Diego, Calif) and median BNP concentration was 165 pg/mL. Seventy-eight probes (of 7370) mapped to 54 unique genes were significantly correlated with BNP concentrations (P<0.001). Of these, the strongest correlated genes (P<0.0001) were in the domains of gelsolin (actin cytoskeleton), matrix metallopeptidases (collagen degradation), platelet function, and immune activity (human leukocyte antigen system, heat shock protein, mast cell, and B-cell lineage).

Conclusions—In the clinically quiescent heart transplant recipient, an elevated BNP concentration is associated with molecular patterns that point to ongoing active cardiac structural remodeling, vascular injury, inflammation, and alloimmune processes. Thus, these findings allude to the notion that BNP elevation is not merely a hemodynamic marker but should be considered reflective of integrated processes that determine the balance between active cardiac allograft injury and repair. (Circulation. 2006;114[suppl I]:I-21–I-26.)

Key Words: gene expression | heart transplantation | natriuretic peptides

B-type natriuretic peptide (BNP), a 32-amino acid neurohormone, secreted predominantly from the cardiac ventricle in response to increased wall stress, is chronically elevated in heart transplant recipients.1 Because an elevation in BNP is associated with cardiopulmonary hemodynamic aberrations, it has been thought to represent diastolic dysfunction or ventriculo-vascular uncoupling of the allograft with its surrounding vasculature.2,3 More recently, investigations have noted that natriuretic peptides in the chronic phase of transplantation are associated with cardiac rejection4 and are predictive of cardiac allograft vasculopathy5,6 and graft loss.7,8

The primary purpose of this investigation included investigation of molecular pathways using peripheral gene expression (GE) patterns that correlate with elevated BNP concentrations in otherwise clinically quiescent heart transplant recipients.

Materials and Methods

Study Design

Of 42 consecutive heart transplant recipients initially screened, we enrolled 28 consecutive clinically quiescent patients in this study at least 4 weeks after heart transplantation who met the inclusion criteria. Clinical quiescence was defined as the absence of any symptoms of dyspnea or fatigue; normal left ventricular ejection fraction (EF >55%) by echocardiography; no histological evidence of rejection on endomyocardial biopsy (International Society for Heart and Lung Transplantation [ISHLT] biopsy score 0 or 1A); and normal hemodynamics (right arterial pressure <7 mm Hg, pulmonary capillary wedge pressure ≤15 mm Hg, and cardiac index ≥2.5 L/min per m²) measured by invasive right heart catheterization. Furthermore, patients with abnormal renal function as defined by a serum creatinine >1.5 mg/dL were excluded because of the possibility of interference with BNP concentrations.8 Clinical data, including immunosuppressive drug regimen and measures of allograft function for each patient encounter, were also collected and analyzed. All patients received tacrolimus and mycophenolate...
motefil-based immunosuppression with adjunctive corticosteroids (prednisone). The study was conducted as part of an Institutional Review Board (IRB)-approved protocol. 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.

**Blood Sampling**

Venous blood samples obtained from enrolled patients were processed on the day of scheduled surveillance biopsies. BNP measurements were performed using whole blood (5 mL) collected into tubes containing potassium EDTA (1 mg/mL blood). The Triage B-type Natriuretic Peptide test (Biosite Diagnostics Inc, San Diego, Calif) was used for this measurement. The Triage BNP test is a fluorescence immunoassay for the quantitative determination of BNP in whole blood and plasma specimens with a turnaround time of 15 minutes and a coefficient of variability of 15%. Peripheral blood mononuclear cells (PBMCs) were isolated from 8 mL venous blood using density gradient centrifugation (CPT tubes; Becton-Dickinson). Samples were frozen in lysis buffer (RLT; Qiagen) within 2 hours of phlebotomy. Total RNA was isolated from each sample (RNeasy; Qiagen) and assessed spectrophotometrically (Spectromax). Two micrograms of RNA from each sample were converted to cDNA, which were then used as templates for in vitro transcription with Cy3-dCTP to generate Cy3-labeled RNA.

**Endomyocardial Biopsy**

Standard techniques were used to obtain biopsy samples, which were graded by local pathologists as well as by 3 independent (“central”) pathologists blinded to clinical information. All samples were obtained >4 weeks after transplant, transfusion, or rejection therapy. Absence of rejection was defined as ISHLT IA or 0 grades, confirmed by a panel of blinded central pathologists.

**Gene Expression**

**Microarray Testing**

A custom microarray was designed using sequences from subtracted, suppressed libraries (25 000 sequences) of stimulated and resting human leukocytes (PCR Select; Clontech) and also identified using publicly available databases; 24 000 50-mer oligonucleotide (Sigma) gene probes (8000 probes in triplicate) representing 7370 genes were spotted on a custom microarray (Telechem). Microarrays were imaged on confocal laser scanners (Agilent) and data were extracted (GenePix 3.0; Axon Instruments), corrected for background and normalized. Gene probes were excluded from analysis if: expression values were available for only ≤10 samples (ie, gene was not detectable in a sufficient number of samples); or the standard deviation of expression values across the samples was <0.05 with expression values expressed as log2(base10)(ratio[sample/control]). This condition was introduced to base correlation analysis on sufficient variance; or average signal-to-noise ratio associated sample was <5 (empirical threshold). This left 5927 gene probes for analysis. Probes mapping to the same gene transcript were not averaged. A global median normalization was applied to each hybridization sample.

**Microarray Data Analysis**

Correlation analysis was performed using Pearson correlation coefficient and rank-based Spearman correlation coefficient. Correlation coefficients (r) and associated P values were computed and averaged for Pearson and Spearman methods. Hierarchical clustering was performed using the pair-wise Pearson correlation coefficients computed from the expression values in the 28 samples as a distance measure. Only gene probes significantly correlated with BNP concentrations were subject to clustering. The average linkage method as implemented in the program OC by Geoffrey Barton was used to generate expression-based dendrograms of gene probes.

**Estimation of the False Discovery Rate**

Gene expression-BNP concentration correlation results in experimental data were compared with randomized BNP concentrations to estimate the proportion of false-positives among the genes identified with P below a certain cutoff value. BNP concentrations were randomly reassigned among the 28 samples and correlation analysis was applied and the process repeated 10 times. The number of probes below a given P value threshold obtained from the randomization trials was then compared with the number of significant genes in the unscrambled data set at the same P value threshold to estimate the proportion of false-positives. As an added precaution for robustness, we implemented the Benjamini and Hochberg frequentist methodology11 for detection of the false discovery rate.

**Results**

**Patients**

The demographic characteristics of the 28 patients enrolled included a mean recipient age of 58±10 years; 68% were men, 96% were white, and it was 16±3 weeks after transplant. The donor characteristics included an average age of 31±6 years; 67% were women and 96% were white. Based on the inclusion criteria of normal hemodynamics, the cohort had a right atrial pressure of 6±1 mm Hg, pulmonary capillary pressure of 11±2 mm Hg and cardiac index of 3.2±0.5 L/min per m². The mean serum creatinine was 1.2±0.1 mg/dL (median, 1.0 mg/dL). The average tacrolimus trough concentration was 12.2±4 ng/mL, whereas prednisone daily dose was 18.9±6 mg. Mycophenolate motefol concentrations were not measured and the average daily dose was 2.4±0.3 grams. These 28 samples on biopsy included 20 with grade 0 and 8 with grade IA pathology as confirmed by the centralized pathologists.

**BNP Concentration**

For the patient cohort, median BNP concentration was 165 pg/mL (mean 255±32 [SEM] pg/mL). Women displayed higher BNP concentrations than men (medians 191 versus 156 pg/mL, P=0.012). There were no correlations of BNP concentration with renal function or hemodynamics in this specially selected cohort.

**BNP and Gene Expression Profiles**

Of the 5927 probes used, 3850 mapped to 2863 unique annotated genes and 2077 did not map to any presently annotated genes. Of the 5927 probes used, 3850 mapped to 2863 unique annotated genes and 2077 did not map to any presently annotated genes; 48 probes were significantly negatively correlated with BNP concentrations (ie, increased gene expression was associated with decreased BNP concentrations), whereas 30 probes were positively correlated with BNP. These 78 gene probes are depicted in the dendrogram with their annotations in the Figure.
Specific Gene Correlates of BNP

We reviewed all uniquely characterized genes (n = 54) and categorized them into domains of cellular remodeling (those encoding proteins involved with cellular structure), vascular injury and repair (those encoding proteins involved with platelet and endothelial function), and alloimmune inflammatory interactions (those genes encoding proteins involved with cellular and humoral immune processes). Additional genes involved in stem cell mobilization pathways, antiviral activity, and apoptosis were also described. These genes and their ascribed functions (n = 25) are detailed in the Table. We did not display those significant genes with a nonspecific function. Several genes (n = 29) encoding cell signaling proteins and G proteins fell into this category. Examples of these genes include PP (inorganic pyrophosphatase), HNR-PDL (heterogeneous nuclear ribonucleoprotein D-like), PDCL3 (phosphducin-like 3), ARHGEF3 (Rho guanine nucleotide exchange factor [GEF] 3), NCL (nucleolin), ELMO2 (engulfment and cell motility 2), HIPK2 (homeodomain interacting protein kinase 2), MPP1 (membrane protein, palmitoylated 1), and CCNF (cyclin F).

Actin cytoskeleton genes (gelsolin) and matrix metalloproteinase genes (MMP8 and MMP9) were strongly upregulated in the presence of elevated BNP concentrations and these genes denote collagen turnover and remodeling at a myocyte, interstitial and vascular level. Gelsolin was increased 25.2-fold (r value 0.74), MMP9 increased 10.5-fold (r value 0.68), and MMP8 increased 6.1-fold (r value 0.67). Genes involved in vascular injury were strongly represented and these included the domains representing platelet function (platelet
<table>
<thead>
<tr>
<th>Action</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Functional Description of Encoded Protein</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remodeling</td>
<td>Gelsolin</td>
<td>GSN</td>
<td>Actin-binding protein involved in remodeling the actin cytoskeleton</td>
<td>Overexpression</td>
</tr>
<tr>
<td></td>
<td>Matrix</td>
<td>MMP9</td>
<td>Breakdown of extracellular matrix in normal physiological and disease processes</td>
<td></td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Metalloproteinases</td>
<td>MMP8</td>
<td>Degrades type IV and V collagens (9) or type I, II, and III collagens (9)</td>
<td>Overexpression</td>
</tr>
<tr>
<td></td>
<td>Endoplasmic reticulum protein 5</td>
<td>ERP5</td>
<td>Regulates binding of fibrinogen, cell surface exposure of P-selectin, and co-association of beta 3 integrin in stimulated platelets.</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Thrombospondin 1</td>
<td>THBS1</td>
<td>Plays a role in platelet aggregation, angiogenesis, and tumorigenesis</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Integrin, Alpha 2b (platelet glycoprotein Iib, antigen CD41B)</td>
<td>ITGA2B</td>
<td>Encodes integrin alpha chain 2b; adhesion, participate in cell surface-mediated signaling</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Platelet factor-4 (chemokine [C-X-C motif] ligand 4)</td>
<td>PF4</td>
<td>Platelet-dependent thrombosis; inhibit T cell proliferation as well as IFN-gamma and IL-2 release; inhibition of angiogenesis and hematopoiesis; promotion of neutrophil</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Plasminogen activator inhibitor-2</td>
<td>PAI-2</td>
<td>Regulator of monocyte proliferation and differentiation; polymorphism of the PAI-2 gene is associated with an increased risk of myocardial infarction</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Thromboxane A2 Receptor</td>
<td>TBXA2R</td>
<td>Thromboxane A2-mediated platelet secretion and aggregation are important in thrombosis and the stable TXA2 analogue, U46619, induces 2 waves of platelet secretion</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Vascular injury and repair</td>
<td>Pro-platelet basic protein (chemokine [C-X-C motif] ligand 7)</td>
<td>PPBP</td>
<td>Platelet-derived growth factor that is a potent chemoattractant and activator of neutrophils</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Inflammation and alloimmune activation</td>
<td>B-cell lymphoma 11B (zinc finger protein)</td>
<td>BCL11B</td>
<td>Translocation may be associated with B-cell malignancies; appears to play a key role in T-cell differentiation</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Inflammation and alloimmune activation</td>
<td>Erythroleukemia virus E26 oncogene homolog 1</td>
<td>ETS1</td>
<td>Genes that are negatively regulated by ETS1 and upregulated by SP100 have anti-inflammatory or antiangiogenic properties; interleukins 2 and 15 regulate Ets1 expression via ERK1/2 and MNK1 in human natural killer cells</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Inflammation and alloimmune activation</td>
<td>Butyrophilin, subfamily 3, member A3</td>
<td>BTN3A3</td>
<td>Involved in the extended major histocompatibility complex</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Inflammation and alloimmune activation</td>
<td>Mast cell-expressed membrane protein 1</td>
<td>MCEMP1</td>
<td>Mast cell transmembrane protein</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Inflammation and alloimmune activation</td>
<td>Signaling lymphocytic activation molecule family member 1</td>
<td>SLAMF1</td>
<td>Activation of peripheral blood cells with agonistic anti-CD3 antibody and exogenous IL-2, as used for generation of cytokine-induced killer cells, results in significant SLAM activation 5 days after T-cell stimulation</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Heat shock 60-kDa protein-1 (chaperonin)</td>
<td>Heat shock 60-kDa protein-1 (chaperonin)</td>
<td>HSPD1</td>
<td>The gene encodes a member of the chaperonin family; the encoded mitochondrial protein may function as a signaling molecule in the innate immune system</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Major histocompatibility complex, class II, DP alpha 1; DR beta 1; DQ alpha 1</td>
<td>Major histocompatibility complex, class II, DP alpha 1; DR beta 1; DQ alpha 1</td>
<td>HLA-DPA1, HLA-DRB1, HLA-DQA1</td>
<td>Plays a central role in the immune system by presenting peptides derived from extracellular proteins</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Histamine receptor H2</td>
<td>Histamine receptor H2</td>
<td>HRH2</td>
<td>Messenger molecule released from mast cells; functionally linked to cellular processes</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Ras homolog gene family, member U</td>
<td>Ras homolog gene family, member U</td>
<td>RHOU</td>
<td>Encodes a member of the Ras family of GTPases; mediates regulation of cell morphology, cytoskeletal organization, and cell proliferation</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Tripartite motif-containing 22</td>
<td>TRIM22</td>
<td>This protein localizes to the cytoplasm and its expression is induced by interferon; function of this protein may be to mediate interferon’s antiviral effects</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Nucleophosmin (nucleolar phosphoprotein B23, numatrin)</td>
<td>NPM1</td>
<td>Involved with upregulation of genes involved in the maintenance of a stem-cell phenotype; may derive from a multipotent hematopoietic progenitor</td>
<td>Overexpression</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Growth arrest and DNA damage-inducible, alpha</td>
<td>GADD45A</td>
<td>Member of a group of genes whose transcript levels are increased after stressful growth arrest conditions and treatment with DNA-damaging agents</td>
<td>Underexpression</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Rho GDP dissociation inhibitor (GDI) beta</td>
<td>ARHGDIB</td>
<td>D4-GDI of Rho family GTPase may be regulated during apoptosis through the caspase-3 mediated cleavage of the GDI protein</td>
<td>Underexpression</td>
</tr>
</tbody>
</table>
factor-4, thromboxane-2, PAI-1), adhesion cell molecules, and integrins (platelet glycoprotein 2B-3A). Platelet factor-4 expression was increased 17.4-fold (r value 0.72). Alloimmune activity related genes were both positively (Histamine Receptor H2 involved in mast cell regulation) and negatively (B cell genes that play a role in T cell differentiation; human leukocyte antigen system genes) correlated with BNP concentrations.

**Discussion**

This investigation has correlated, for the first time to our knowledge, gene expression pathways associated with increased expression of BNP in the clinically quiescent phase of heart transplantation. Contrary to common perception that BNP is a neurohormone principally responsive only to hemodynamic perturbations, we have confirmed that an elevation of this peptide during normal hemodynamics and in the absence of histological allograft rejection, reflects upregulation of molecular pathways representing ongoing inflammation, alloimmune activation, cardiac structural remodeling, and vascular perturbations. These observations lend credence to the notion that BNP elevations in the quiescent phase after heart transplantation are reflective of ongoing allograft injury and remodeling at activity levels not discernable with clinical techniques. This may explain observations from studies that have found this biomarker to possess prognostic predictive power for the development of cardiac allograft vasculopathy and allograft failure.

The strong association of the gelsolin gene and matrix metalloproteinase genes with an elevated BNP point to the important regulatory role of this natriuretic peptide in cardiac structural remodeling. The gelsolin gene family encodes a number of actin-binding proteins that are thought to function in the cytoplasm by severing, capping, nucleating, or bundling actin filaments. Others have demonstrated that the binding step of collagen phagocytosis is facilitated by Ca(2+)/H11001 binding to long-term survival in heart transplantation. The allograft vasculopathy and this entity is the strongest limitation to long-term survival in heart transplantation.

The genesis of cardiac allograft vasculopathy is determined by both alloimmune and nonimmunological factors but inflammation is central in its development. Thrombosis and inflammation are intertwined in the development of vascular disease. Platelet factor 4 (PF-4), a member of the CXC subfamily of chemokines, is secreted in high amounts by activated platelets. PF-4 specifically binds to human polymorphonuclear granulocytes, but requires tumor necrosis factor alpha-α as a costimulus for the induction of effector functions in vascular cells. It has been demonstrated that long-term survivors of heart transplantation are characterized by activation of platelets, which contain increased levels of soluble CD-40 and release enhanced cytokines when stimulated. Animal studies have shown that lack of plasminogen activator inhibitor-1 expression in donor tissue greatly exaggerates the extent of vascular intimal proliferation after allogeneic transplantation. Similarly, integrins are cell surface adhesion receptors that mediate cell–cell and cell–extracellular matrix interaction with several ligands, including fibrinogen, fibronectin, thrombospondin, and prothrombin. Myocardial ischemic injury after cardiac transplantation is associated with upregulation of integrins, tissue factor, and activation of the MMP induction system, which may contribute to the subsequent development of allograft remodeling and vasculopathy. Thrombospondin-1, a matrix glycoprotein, inhibits angiogenesis and facilitates smooth muscle cell proliferation. Increased levels of thrombospondin-1 in human cardiac allografts may alter vascular responses to angiogenic growth factors by inhibiting angiogenesis and promoting smooth muscle cell proliferation characteristic of cardiac allograft vasculopathy. Our investigation suggests that BNP elevation is indicative of upregulation of pathways in thrombosis, inflammation, and vascular remodeling in heart transplantation. Importantly, the strong expression of genes regulating platelet function points to the need for anti-platelet therapeutic studies in heart transplantation.

Although we enrolled a selective subset of patients without overt rejection as evidenced by normal allograft function and absence of histological rejection, our investigation correlated genes in the mast cell function domain with BNP elevation. Human mast cells, by elaborating various cytokines, chemokines, and proinflammatory mediators play a complex role in inflammatory disorders and have been linked with cardiac rejection. However, in post-transplant hearts during ongoing acute rejection, the mast cells are phenotypically different from nonrejecting hearts and isografts. Others have shown that mast cells are not necessarily associated with rejection but underlie enhanced inflammation, neovascularization, and fibrosis during cardiac allograft arteriosclerosis. Thus, in our study, it is more likely that the correlation of mast cell genes reflects ongoing inflammation rather than a signature for allograft rejection.

Finally, we acknowledge several limitations of this work. First, the patient number is small; however, we chose to develop a highly selected cohort of clinically quiescent patients with normal renal function within the first year of transplant and in this context represents a reasonable sample size. Second, we correlated peripheral blood gene expression patterns with BNP concentrations and did not evaluate
intragraft events. Therefore, it should be recognized that overexpression or underexpression of a specific gene in peripheral blood should not necessarily be construed to describe the direction of that pathway within the allograft. Third, we used a custom leukocyte microarray and it is entirely possible that we might have missed some significant pathways if a whole genome approach had been undertaken. However, the fact that a number of genes that correlated with BNP concentrations all mapped to the same domain of molecular pathways increases the likelihood of the robustness of our findings. We concede that independent validation studies should help confirm these findings.

Conclusion
In the clinically quiescent heart transplant recipient, an elevated BNP concentration is associated with molecular patterns that point to ongoing active cardiac structural remodeling, vascular injury, inflammation, and alloimmune processes. Thus, these findings allude to the notion that BNP elevation is not merely a hemodynamic marker but should be considered reflective of integrated processes that determine the balance between active cardiac allograft injury and repair.

Acknowledgment
The authors express their gratitude to Steve Gottlieb, MD, for his critical appraisal of this manuscript.

Sources of Funding
This study was supported in part by a research grant from XDx Inc. M.R.M. has received research grants from XDx Inc.

Disclosures
M.R.M. is a consultant for XDx, Inc and D.W., J.G.W., J.P., and D.T. are or were employees of XDx Inc.

References
Gene Expression Profiles and B-Type Natriuretic Peptide Elevation in Heart Transplantation: More Than a Hemodynamic Marker
Mandeep R. Mehra, Patricia A. Uber, Dirk Walther, Mark Vesely, Jay G. Wohlgemuth, James Prentice, Darren Tayama and Margaret Billingham

*Circulation*. 2006;114:I-21-I-26
doi: 10.1161/CIRCULATIONAHA.105.000513

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 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/114/1_suppl/I-21

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/