Basic Science Reports

Intracoronary Adenovirus-Mediated Delivery and Overexpression of the β2-Adrenergic Receptor in the Heart

Prospects for Molecular Ventricular Assistance

Ashish S. Shah, MD; R. Eric Lilly, MD; Alan P. Kypson, MD; Oliver Tai, BS; Jonathan A. Hata, BA; Anne Pippen, BS; Scott C. Silvestry, MD; Robert J. Lefkowitz, MD; Donald D. Glower, MD; Walter J. Koch, PhD

Background—Genetic modulation of ventricular function may offer a novel therapeutic strategy for patients with congestive heart failure. Myocardial overexpression of β2-adrenergic receptors (β2-ARs) has been shown to enhance contractility in transgenic mice and reverse signaling abnormalities found in failing cardiomyocytes in culture. In this study, we sought to determine the feasibility and in vivo consequences of delivering an adenovirus containing the human β2-AR cDNA to ventricular myocardium via catheter-mediated subselective intracoronary delivery.

Methods and Results—Rabbits underwent percutaneous subselective catheterization of either the left or right coronary artery and infusion of adenoviral vectors containing either a marker transgene (Adeno-βGal) or the β2-AR (Adeno-β2AR). Ventricular function was assessed before catheterization and 3 to 6 days after gene delivery. Both left circumflex- and right coronary artery–mediated delivery of Adeno-β2AR resulted in ∼10-fold overexpression in a chamber-specific manner. Delivery of Adeno-βGal did not alter in vivo left ventricular (LV) systolic function, whereas overexpression of β2-ARs in the LV improved global LV contractility, as measured by dP/dt max, at baseline and in response to isoproterenol at both 3 and 6 days after gene delivery.

Conclusions—Percutaneous adenovirus-mediated intracoronary delivery of a potentially therapeutic transgene is feasible, and acute global LV function can be enhanced by LV-specific overexpression of the β2-AR. Thus, genetic modulation to enhance the function of the heart may represent a novel therapeutic strategy for congestive heart failure and can be viewed as molecular ventricular assistance. (Circulation. 2000;101:408-414.)

Key Words: gene therapy ▪ myocardium ▪ receptors, adrenergic, β ▪ ventricles ▪ heart failure ▪ signal transduction

Novel treatment strategies for ventricular dysfunction are of great importance, and the area of gene therapy is gaining increasing attention. Traditional approaches to the treatment and palliation of ventricular dysfunction and failure include medical management,1 myocardial revascularization,2 valve repair,3 and mechanical assist and transplantation.4 Although there have been advances in these therapies, congestive heart failure (CHF) remains a leading cause of death in the United States and worldwide.5 Thus, a molecular approach for treating the failing heart is attractive. Early work in cardiac gene therapy has concentrated on 2 distinct problems: reliable methods of delivery to working myocardium and identification of potential molecular targets. First, work by several groups demonstrated the feasibility of delivering transgenes via direct intramyocardial injection,6,7 ex vivo perfusion,8,9 and finally a transluminal intracoronary approach.10 Second, a spectrum of molecular targets has emerged through work done in genetically engineered mice.11 One molecular target identified is the overexpression of the β2-adrenergic receptors (β2-ARs). Transgenic mice with cardiac-specific overexpression of the human β2-AR at either >100-fold over endogenous β2-AR density12 or significantly lower overexpression13 have enhanced contractility without overt pathological conditions. These models were developed to replace receptors that are lost during the development of CHF. In the failing heart, there is a 50% reduction of myocardial β-adrenergic receptors (β-ARs), with remaining receptors being functionally uncoupled.14 In addition to the positive phenotype of the β2-AR-overexpressing mice, adenovirus-mediated overexpression of β2-ARs in failing rabbit ventricular cardiomyocytes in culture has resulted in the functional rescue of the signaling abnormalities present in failing heart cells.15 Thus, genetically replacing lost β-ARs in the failing heart represents a potentially novel therapeutic strategy to increase inotropy.

Received April 30, 1999; revision received July 29, 1999; accepted August 11, 1999.


Correspondence to Walter J. Koch, PhD, Laboratory of Molecular Cardiovascular Biology, Box 2606, MSRB Room 471, Duke University Medical Center, Durham, NC 27710. E-mail koch0002@mc.duke.edu

© 2000 American Heart Association, Inc.

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

408
The primary hurdle to testing the feasibility of $\beta_2$AR gene transfer in vivo is the development of a clinically relevant gene delivery system. Recent work by our laboratory and others has shown that it is possible to deliver transgenes globally to the myocardium by adenoviral vectors. In these studies, adenoviruses were delivered via a surgically invasive approach in which the transgenes are injected into the left ventricular (LV) cavity while the aorta is cross-clamped, directing the adenoviral solution to perfuse the coronary arteries. Using this method, we showed that overexpression of $\beta_2$ARs in the rabbit heart does enhance global in vivo LV function. Because this method of gene delivery has its limitations, we explored the feasibility of delivering the $\beta_2$AR transgene to the rabbit heart in vivo via percutaneous subselective coronary catheterization and injection. A previous report showed that transluminal intracoronary artery delivery of marker transgenes in the rabbit is possible; however, that report did not include a study of myocardial function. The purpose of our study was to develop a reproducible percutaneous subselective intracoronary artery delivery method for efficient ventricle-targeted in vivo gene transfer of adenoviral transgenes to rabbit myocardium. Furthermore, we investigated whether ventricular overexpression of the $\beta_2$AR in the rabbit heart could alter biochemical and in vivo cardiac function.

Methods

Adenoviral Constructs

Adenoviral constructs using a “first-generation” E1/E3-deleted replication-deficient adenovirus have been described previously. The $\beta_2$AR construct (Adeno-$\beta_2$AR) and the marker transgene $\beta$-galactosidase (Adeno-βGal) were driven by the CMV promoter. Large-scale preparations of these adenoviruses were purified from infected Epstein-Barr nuclear antigen–transfected 293 cells as described. Adenoviral constructs using a “first-generation” E1/E3-deleted replication-deficient adenovirus have been described previously. The $\beta_2$AR construct (Adeno-$\beta_2$AR) and the marker transgene $\beta$-galactosidase (Adeno-βGal) were driven by the CMV promoter. Large-scale preparations of these adenoviruses were purified from infected Epstein-Barr nuclear antigen–transfected 293 cells as described. Adenoviral constructs using a “first-generation” E1/E3-deleted replication-deficient adenovirus have been described previously. The $\beta_2$AR construct (Adeno-$\beta_2$AR) and the marker transgene $\beta$-galactosidase (Adeno-βGal) were driven by the CMV promoter. Large-scale preparations of these adenoviruses were purified from infected Epstein-Barr nuclear antigen–transfected 293 cells as described.

Percutaneous Intracoronary Adenovirus Delivery and Cardiac Functional Assessment

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the NIH. Forty-seven adult male New Zealand White rabbits (3 kg) were used in the present study. At the time of the initial study, animals were sedated with ketamine (50 mg/kg) and acepromazine (0.25 mg/kg), and an incision was made over the right neck to expose the right carotid and jugular vessels. A 2.5F micromanometer (Millar Inc) was placed into the LV cavity via the carotid artery under fluoroscopic guidance, and a 22-gauge angiocatheter was placed into the jugular vein. The micromanometer was coupled to a PC-based data acquisition system (Physiological Systems Inc, and LV pressure was obtained at baseline and after the infusion of isoproterenol at 0.5 μg · kg$^{-1}$· min$^{-1}$ for 20 minutes as described. All hemodynamic studies were performed in a closed-chest model with an intact pericardium. After ventricular functional assessment of individual rabbits, a 4.5F radial arterial line sheath (Cook Inc) was placed into the carotid artery, and a 3F coronary catheter (Cook Inc) was placed into the left circumflex (LCx, n = 37) or right coronary artery (RCA, n = 10) under fluoroscopic guidance to deliver the adenoviral transgenes. The coronary catheter used to perform these subselective coronary artery adenovirus injections was a simple right-angle catheter with a single distal port (Cook Inc). Before virus infusion, adenosine (1.5 mg), lidocaine (0.33 mg), and heparin (500 U) were given via the jugular vein, and $5 \times 10^{10}$ total viral particles (TVP) of a replication-deficient adenovirus in 2.5 mL of PBS at 37°C was injected as a bolus into the respective coronary artery. In addition to adenovirus, a subset of animals (n = 4) received only PBS as a control for the delivery technique. All animals were returned to their cages after they had recovered and were fully alert. In 3 days, cardiac hemodynamics of rabbits that received adenovirus or saline were studied as above. Thus, each rabbit served as its own control for cardiac function. All rabbits received methylprednisolone 5.0 mg · kg$^{-1}$ · d$^{-1}$ IM for 2 days after adenovirus delivery. After the final assessment, animals were euthanized and their hearts rapidly excised. Transmural samples of the LV free wall, right ventricle (RV), septum, left atrium, lung, brain, and liver were frozen in liquid nitrogen and stored for biochemical analysis.

To examine the effects of heart rate (HR) on maximal rate of pressure rise, rabbits (n = 4) underwent transvenous atrial pacing via the right jugular vein, and LV pressure was measured as described above. HR was varied from baseline to 330 bpm.

Data Analysis of In vivo Cardiac Function

Analog data were digitized at 200 Hz and analyzed on a VAX workstation (Digital Equipment Corp) with custom software (Physiological Systems Inc). The maximal rate of pressure rise (dP/dt max) was computed from the digital pressure waveform as a running 5-point polyorthogonal transformation. All hemodynamic data were derived from the average of 20 steady-state cardiac cycles.

$\beta$-AR Density

$\beta$-AR binding was performed on myocardial sarcolemmal membrane preparations as we have previously described. Total myocardial $\beta$-AR density was determined by incubating 25 μg of sarcolemmal membranes with a saturating concentration of $^{125}$I-labeled cyanopindolol and 20 μmol/L alprenolol to define nonspecific binding. Assays were performed in triplicate, and $\beta$-AR density was normalized to milligrams of membrane protein.

$\beta$-Galactosidase Staining

After excision, transverse cross sections of myocardium at the midpapillary level were obtained for histological analysis and stored in 30% sucrose solution before paraffin embedding as described. Paraffin-embedded samples were mounted on a cryostat and sectioned into 5- to 10-μm sections, which were then transferred to a glass slide. $\beta$-Gal staining was performed by standard procedures as described.

$\beta_2$AR Immunohistochemistry

Frozen myocardial sections were cut at 10 μm for indirect immunofluorescence studies as we have described. Briefly, sections were rinsed in PBS and then in PBS with 0.05% Triton X-100 (Triton-PBS, blocked with serum diluent and 0.1% BSA and 0.1% sodium azide), and then rinsed for 15 minutes in Triton-PBS before overnight incubation at 4°C with a primary rabbit anti-human $\beta_2$AR antiserum (1:500 dilution in serum diluent). The sections were then washed, incubated for 1 hour in FITC-conjugated goat anti-rabbit immunoglobulin G (1:50 dilution in serum diluent), rinsed in PBS, mounted with sodium iodide (25 g/L) in 1:1 PBS/glycerol solutions, and photographed.

Statistical Analysis

All data are expressed as the mean±SEM. In vivo hemodynamic data were compared by a paired Student’s t test. Unpaired comparisons were made by use of a 1-way ANOVA. For all analyses, a value of P<0.05 was considered to be statistically significant.

Results

Adenovirus-Mediated Myocardial Overexpression of Transgenes After Intracoronary Delivery

We delivered adenoviral transgenes via either the LCx or RCA in a subselective manner. Transgene expression in the rabbit myocardium was assessed 3 days after delivery to...
allow time for adequate protein translation and before adeno-
viral immunological processes were evident. As further
protection toward the latter, we treated rabbits (including all
control groups) for 2 days with corticosteroids.

To assess the volume of myocardium expressing transgene,
we delivered Adeno- βGal (5×10^{11} TVP) via either the LCx
or RCA. Figure 1 shows representative X-Gal–stained cardiac
cross sections at low and high power of magnification after

Adeno- βGal delivery. Chamber-specific expression of the
βGal gene that corresponded to staining in individual myo-
cytes was evident (Figure 1). Thus, it appears that within the
region served by the catheterized coronary artery, complete
transmural expression is possible, especially in this adenovi-
dral dose range.

We also studied the delivery and expression of the human
β2 AR, which represents a potentially therapeutic transgene.
As with Adeno- βGal, we delivered 5×10^{11} TVP Adeno-
β2 AR via either the LCx or RCA and initially assessed β-AR
density. As shown in Figure 2A, Adeno- β2 AR delivery in the
LCx resulted in significant LV-specific overexpression,
whereas catheterization and injection via the RCA results in
RV-specific expression. β2 AR overexpression ranged from
≈9- to 15-fold over endogenous myocardial β-AR density.
Expression of the β2 AR transgene remained elevated at 6
days (n=4) after gene delivery (Figure 2B). Importantly,
β2 AR overexpression after in vivo coronary delivery was
found to be localized to the sarcolemmal membranes of
individual ventricular myocytes as visualized by immunohis-
tochemical staining with an antibody specific for the human
β2 AR (Figure 3). As with the βGal transgene distribution,
β2 AR overexpression found by immunohistochemistry was
diffuse throughout the entire LV after LCx injection, and a
representative section of an Adeno- β2 AR–treated LV is
shown in Figure 3.
Functional Consequences of Subselective Intracoronary Adenovirus Delivery and Myocardial Overexpression of Adeno-βGal and Adeno-β2AR Transgenes

The hemodynamic consequences of subselective intracoronary delivery of adenoviral transgenes were examined by use of dynamic intracavitary pressure measurements. The maximal first derivative of the LV intracavitary pressure (dP/dt max) was used as a measure of global LV contractile performance. As described in the Methods, the hemodynamics of each rabbit in the study was measured before and then 3 to 6 days after gene delivery. Thus, each animal served as its own control, increasing the power of the analysis. Furthermore, animals that underwent catheterization and injection of saline served as controls for the delivery technique itself. β-AR density determined for all animals in the study confirmed β2AR overexpression. Animals that received Adeno-βGal via the LCx did not show a significant change in systolic function compared with both precatheterization values and animals injected with saline (Figure 4A). In animals that received Adeno-β2AR and subsequently showed β2AR overexpression in the LV, a significant increase in baseline dP/dt max was seen compared with precatheterization values and Adeno-βGal–treated animals (Figure 4B). LV systolic functional responses to isoproterenol were also significantly greater in β2AR-overexpressing animals compared with controls (Figure 4C).

Interestingly, in animals that received either Adeno-β2AR or Adeno-βGal (5×10¹¹ TVP each), HRs and LV end-diastolic pressure (EDP) were significantly increased 3 days after gene delivery, whereas there was no change, with either treatment, in systolic blood pressure (Table 1). LV dP/dt max in Adeno-βGal–treated rabbits was significantly reduced, whereas no reduction was evident in Adeno-β2AR–treated rabbits (Table 1). Hemodynamic values were not altered 3 days after saline delivery including dP/dt max (Figure 4A), dP/dt min (before, −2387±148 mm Hg/s versus after, −2152±196 mm Hg/s, P=NS), and LV EDP (before, 0.7±0.15 mm Hg versus after, 0.9±0.4 mm Hg, P=NS).

LV dP/dt max measurements can be confounded by changes in HR and afterload. Accordingly, a subset of animals was studied to determine the effect of HR on LV dP/dt max by use of transvenous atrial pacing. As shown in Figure 5, the HR effect in the range of 200 to 300 bpm did not significantly increase LV dP/dt max in a normal rabbit, demonstrating that increased HR is not accountable for the significant increase in basal and isoproterenol-stimulated LV dP/dt max found in Adeno-β2AR–treated rabbits.
The functional benefit of β2AR overexpression in the LV was also studied 6 days (n=4) after gene delivery. Importantly, basal and isoproterenol-stimulated LV systolic performance remained elevated at 6 days compared with pre–gene delivery hemodynamic measurements (Table 2).

Finally, the effect of β2AR overexpression in the RV after Adeno-β2AR delivery via the RCA on LV function was also studied. No improvement in LV systolic performance was seen in animals that overexpressed the β2AR in the RV (n=6) from precatheterization baseline values (before catheterization, 2725.8±211 versus after catheterization, 2858.7±48 mm Hg/s, P=NS).

**Discussion**

The present study reports the novel findings that in vivo molecular manipulation of ventricular function by a percutaneous intracoronary gene delivery method is feasible. Accordingly, we have demonstrated that chamber-specific delivery of an adenovirus containing the human β2AR via subselective coronary catheterization and injection produces ventricular overexpression of the β2AR at a level that increases contractile function in vivo, demonstrating a therapeutic strategy to effectively produce molecular ventricular assistance in CHF.

The β2AR signaling system is an appealing target for cardiac gene therapy, because specific molecular abnormalities have been well described in human CHF and in multiple animal models of failure and cardiac disease. A down-regulation of β-ARs, uncoupling from second messenger systems, and elevation of desensitizing G-protein receptor kinase activity are the fundamental alterations seen in heart failure. In myocytes isolated from hearts in CHF, these abnormalities can be reversed by β2AR overexpression or G-protein–coupled receptor kinase inhibition by use of transgenes delivered via adenoviral vectors. These studies, as well as studies in transgenic mice, form the basis of our β2AR gene therapy strategy.

The overexpression of β2ARs in the LV free wall appears to improve global LV systolic performance. dP/dt max both at baseline and after isoproterenol administration was increased relative to the precatheterization values in each of the individual rabbits. This is consistent with our findings in a model of biventricular overexpression of β2ARs. It is extremely encouraging that the ≈20% improvement seen in this study occurred after only LV free wall transfection and a relatively modest 10-fold increase in LV β2AR density.

Furthermore, this functional benefit was sustained at 6 days after gene delivery. Previous reports in transgenic mice demonstrated greater improvement in LV contractile function with β2AR overexpression. However, these mice had considerably higher levels of β2AR overexpression, which was global in nature and not limited to a single chamber, as is the case in the rabbits of this study. This is the first animal model to examine the in vivo effects of chamber-specific overexpression of the β2AR in the intact circulation, where ventricular interactions limit the maximal improvement in performance. Rabbits treated with Adeno-βGal did not show a significant difference in systolic function compared with saline-injected animals. This demonstrates that adenovirus

### Table 1. Summary of Hemodynamic Data Before (Pre-Cath) and 3 Days After (Post-Cath) Intracoronary LCx Delivery of Adeno-β2AR (n=10) or Adeno-βGal (n=10) (5×10¹⁴ TVP)

<table>
<thead>
<tr>
<th></th>
<th>Adeno-βGal</th>
<th>Adeno-β2AR</th>
<th>Adeno-β2AR</th>
<th>Adeno-βGal</th>
<th>Adeno-β2AR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LV dP/dtmax, mm Hg/s</strong></td>
<td>2998.8±163</td>
<td>2911.5±188</td>
<td>2837.2±213</td>
<td>3454.8±237*</td>
<td></td>
</tr>
<tr>
<td><strong>LV dP/dtmin, mm Hg/s</strong></td>
<td>245±8.3</td>
<td>244±6.2</td>
<td>275±13*</td>
<td>278±8*</td>
<td></td>
</tr>
<tr>
<td><strong>HR, bpm</strong></td>
<td>245±8.3</td>
<td>244±6.2</td>
<td>275±13*</td>
<td>278±8*</td>
<td></td>
</tr>
<tr>
<td><strong>LV EDP, mm Hg</strong></td>
<td>0.7±0.28</td>
<td>0.25±0.39</td>
<td>2.6±0.7*</td>
<td>5.8±1.4*</td>
<td></td>
</tr>
<tr>
<td><strong>LV SBP, mm Hg</strong></td>
<td>67.1±2.5</td>
<td>69.5±1.8</td>
<td>64±2.5</td>
<td>72.4±2.7</td>
<td></td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure.

*P<0.05 vs Pre-Cath.

### Table 2. Summary of In Vivo LV Performance Before (Pre-Cath) and 6 Days After (Post-Cath) Adeno-β2AR Gene Delivery (n=4)

<table>
<thead>
<tr>
<th></th>
<th>Pre-Cath</th>
<th>Post-Cath (6 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dP/dtmax, mm Hg/s</strong></td>
<td>2833.2±174</td>
<td>3593.7±307*</td>
</tr>
<tr>
<td><strong>dP/dtmin, mm Hg/s</strong></td>
<td>-2664.2±109</td>
<td>-2802.0±200</td>
</tr>
<tr>
<td><strong>Isoproterenol-stimulated dP/dtmax, mm Hg/s</strong></td>
<td>4646.4±242</td>
<td>5149.8±384†</td>
</tr>
</tbody>
</table>

*P<0.05 vs Pre-Cath; †P=0.10 vs Pre-Cath.
delivery, and transgene expression in general, does not depress baseline dP/dt_{max}. Interestingly, β₂AR overexpression directed to the RV did not enhance LV performance. Although this result is not surprising, RV delivery of β₂ARs may offer benefit in conditions of isolated right-sided dysfunction such as pulmonary hypertension.

We did observe a degree of myocardial injury with this model. In Adeno-βGal controls and β₂AR-treated rabbits, EDP was elevated and Adeno-βGal–treated rabbits had depressed dP/dt_{max} values. Saline-injected controls did not show alterations of EDP or dP/dt_{max}. Overall, these data suggest that bolus delivery of adeno virus caused an increase in LV stiffness. Interestingly, animals that overexpressed the β₂AR had less depression of dP/dt_{max} at 3 and 6 days, suggesting a superimposed lusitropic effect. Nevertheless, this apparent negative effect of intracoronary adenovirus injection needs to be examined further.

The present study highlights a number of challenges to in vivo cardiac gene therapy. First, myocardial delivery of the adenoviral transgene has been achieved in this percutaneous model, but whether the methods used here will be applicable to other viral vectors is not certain. The kinetics of adenovirus-mediated transfer in the intact coronary vasculature favor high coronary perfusion flow and pressure as well as enhanced permeability,20 most of which can induce myocardial injury. We use a relatively large injection volume for individual coronary arteries, which we have found leads to significant improvement in gene transfer and transgene expression, consistent with the ex vivo kinetic studies of intracoronary adenoviral gene delivery.20 Furthermore, the present study uses higher concentrations of virus than previously reported; however, transmural myocardial expression is also greater than in previous reports.10 Accordingly, a significant percentage of ventricular myocytes must be transfected to achieve a global functional impact.

The duration of transgene expression was not examined in this study. Work in our laboratory has documented that the first-generation vectors used yield expression that lasts for 1 to 2 weeks in vivo in the myocardium.9,16 However, we have clearly shown that adenovirus-mediated transgene delivery can alter the in vivo function of the heart for ≥1 week, and the use of this method can only improve in the future as vector technology advances to allow for less inflammatory vectors that also support longer-term expression. For example, long-term myocardial expression has recently been reported with adenov-associated virus.21 It will be of particular future interest to determine whether our delivery method will effectively deliver adeno-associated virus transgenes.

There are several implications of our experimental findings. The present study is among the first to demonstrate the feasibility of cardiac gene transfer by clinically relevant and available methods. Previous studies have used open thoracotomy and aortic cross-clamping.16,17 which would not be as widely useful to the 4 million patients with CHF. We have also demonstrated the ability to enhance baseline myocardial function in the otherwise normal LV. This improvement of baseline systolic function is consistent with previous findings in β₂AR-overexpressing transgenic mice,12,13 including transgenic mice that had myocardial-targeted β₂AR overexpres-

**References**


Intracoronary Adenovirus-Mediated Delivery and Overexpression of the β₂-Adrenergic Receptor in the Heart: Prospects for Molecular Ventricular Assistance
Ashish S. Shah, R. Eric Lilly, Alan P. Kypson, Oliver Tai, Jonathan A. Hata, Anne Pippen, Scott C. Silvestry, Robert J. Lefkowitz, Donald D. Glower and Walter J. Koch

Circulation. 2000;101:408-414
doi: 10.1161/01.CIR.101.4.408

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 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/101/4/408

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/