Gene Therapy for Attenuating Cardiac Allograft Arteriopathy Using Ex Vivo E2F Decoy Transfection by HVJ-AVE–Liposome Method in Mice and Nonhuman Primates

Motohiro Kawauchi,* Jun-ichi Suzuki,* Ryuichi Morishita, Yuko Wada, Atsushi Izawa, Naruya Tomita, Jun Amano, Yasufumi Kaneda, Toshio Oghara, Shinichi Takamoto, Mitsuaki Isobe

Abstract—Cardiac allograft arteriopathy, which limits the long-term survival of recipients, is characterized by diffuse intimal thickening composed of proliferative smooth muscle cells. The transcription factor E2F plays a pivotal role in the coordinated transcription of cell-cycle regulatory genes. To test the hypothesis that double-stranded DNA with specific affinity for E2F (E2F decoy) is effective in preventing intimal hyperplasia, we performed ex vivo single intraluminal delivery of E2F decoy into cardiac allografts of mice and Japanese monkeys using the hemagglutinating virus of Japan (HVJ) artificial viral envelope–liposome method. In murine models, antisense cyclin-dependent kinase 2 (cdk2) kinase oligodeoxynucleotide (ODN) and no transfers were performed to compare the effects. Severe intimal thickening was observed, and multiple cell-cycle regulatory genes were enhanced in untreated allografts. E2F decoy prevented neointimal formation and suppressed these genes for up to 8 weeks, whereas antisense cdk2 kinase ODN had limited effects. In primate models, E2F decoy dramatically prevented neointimal thickening and suppressed multiple cell-cycle regulatory genes, whereas intimal thickening developed in the nontransfected or mismatch decoy-transfected allografts. Gel mobility shift assay proved the specific effects of E2F decoy, and reverse transcriptase–polymerase chain reaction documented that neither complication nor dissemination of HVJ into other organs was observed. We demonstrate that ex vivo gene delivery to allografts is a potent strategy to modify allograft gene expression, resulting in prevention of graft arteriopathy without systemic adverse effects. (Circ Res. 2000;87:1063-1068.)

Key Words: primate ■ heart transplantation ■ transcription factor ■ arteriosclerosis ■ gene therapy

Cardiac transplantation often results in accelerated graft coronary disease in long-term survivors.1 Neointimal hyperplasia of the coronary arteries results from vascular smooth muscle cell (SMC) proliferation; this proliferation is dependent on the coordinated actions of cell-cycle regulatory genes.2 Recently, we reported that antisense cyclin-dependent kinase (cdk) 2 kinase oligodeoxynucleotide (ODN) prevents neointimal formation in murine cardiac allografts.3 The transcription factor E2F regulates multiple cell-cycle regulatory genes, which are critical to the process of cell growth and proliferation.4,5 Double-stranded DNA with high affinity for E2F acting as a decoy (E2F decoy) inhibits cell-cycle regulatory gene expression and SMC proliferation in rat carotid injury models.6 However, the effect of E2F decoy in preventing graft coronary arterial neointimal formation has not been investigated. Several gene therapy trials have been performed in search of methods to prevent and treat several diseases using the hemagglutinating virus of Japan (HVJ)-liposome method.7 Recently, HVJ artificial viral envelope (AVE) liposome, which is a modified method of HVJ-liposome delivery, has been demonstrated to increase efficiency of cellular uptake of ODN.8 Although many innovative and attractive results have been reported using this strategy, there have been no reports on the efficacy and safety of double-stranded E2F decoy ex vivo transfection into cardiac allografts. In this study, we revealed, using murine and primate models, that ex vivo gene delivery to allografts is a potent strategy to modify allograft gene expression, resulting in prevention of graft arteriopathy without systemic adverse effects.

Materials and Methods
In murine models, DBA/2 hearts were transplanted heterotopically into B10.D2 mice as allografts. The HVJ-AVE liposome, phosphorothioate decoy, and ODN were prepared and were infused into the allografts ex vivo during the interval between donation and implantation.3–6 E2F decoy or antisense cdk2 kinase ODN was transfected...
into the allografts; control donor hearts did not receive any gene transfection (n=12 for each group). Immediately after transfection, donor hearts were heterotopically transplanted into recipient mice using a microsurgical technique.5,9,10 The grafts were harvested on days 28 and 56 after transfection.

In primate models, Japanese monkeys were used for cardiac transplantation.11,12 FITC-labeled decoy was injected to confirm the transfection. E2F decoy (n=5) or mismatch decoy (n=5) was transfected into the allografts; control donor hearts did not receive any gene transfection (n=4). Donor hearts were transplanted, and the recipients were treated with FK506 (Fujisawa Pharmaceutical Co.).

The allografts were harvested at day 28.11

For pathological analysis, murine and primate cardiac allografts were sectioned and stained with Elastica van Gieson (EvG) to highlight the internal elastic lamina (IEL). The area was calculated according to the following formula: luminal occlusion=(IEL area−luminal area)/IEL area. Myocardial rejection was scored using myocardial sections.3 For immunohistochemistry, allograft sections were incubated with primary antibodies (proliferating-cell nuclear antigen [PCNA], cdk2, c-myb, c-myc [Santa Cruz Biotech], and SMemb [Yamasa]) for 12 hours at 4°C.4 Antibody-biotin conjugate was detected with Vectastain ABC kit (Vector) and scored as previously described.3,12 Using in situ reverse transcriptase–polymerase chain reaction (RT-PCR), expression of PCNA, cdc2, and cdk2 mRNA was examined.3,13 Briefly, RT solution with 3′ primer was applied to each slide, which was then incubated at 42°C for 15 minutes. These sections were then incubated with PCR solution including 3′ and 5′ primers with the following parameters: 94°C, 1 minute; 55°C, 2 minutes; and 72°C, 1 minute; 35 cycles.

For detecting HVJ in primate systemic organs, we performed RT-PCR with the primers for HVJ F and HN protein.14,15 RNA was extracted from primate cardiac allografts and systemic recipient organs removed at day 28 from HVJ-AVE liposome complex-transfected allograft recipients. RNA derived from organs was amplified using RT-PCR (35 cycles) and compared with positive (using RNA from noninactivated HVJ and recipient liver) and negative controls (primers without RNA). β-Actin was used as an internal control.6

To prove the specific effect of E2F decoy, we performed a gel mobility shift assay. Briefly, nuclear extract was prepared from primate cardiac allografts. E2F decoy phosphorothioate ODN primer was 32P-labeled and incubated for 30 minutes and then loaded onto a 4% polyacrylamide gel. The gels were subjected to electrophoresis and dried.6

All quantitated data were analyzed as mean±SD and compared among the groups using Scheffe’s ANOVA.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

### Results

#### Murine Models

**Histological Findings of the Graft Arteries**

All isografts and allografts kept beating throughout the observation period. Coronary arteries of native hearts and isografts did not develop intimal thickening during this study. At 28 days after transplantation, heavy neointimal thickening was observed in the coronary arteries of untreated allograft recipients. In recipients treated with antisense cdk2 kinase ODN or E2F decoy, arterial intima was mildly thickened. These values did not differ significantly between the antisense and decoy treatment groups; however, they were significantly less than values in the nontreated group. In the E2F decoy recipients 56 days after transplantation, arterial intimal thickening was dramatically prevented. Intimal thickening scores in allografts treated with E2F decoy did not progress from day 28 to day 56. There was a significant difference in the degree of intimal thickening between E2F decoy, antisense cdk2 kinase ODN, and nontreated allografts at day 56. Cell infiltration in myocardium did not differ among the groups at days 28 and 56 (Table 1, Figure 1).

**Cell-Cycle Regulatory Gene Expression on Graft Arteries**

Isografts did not express PCNA, c-myb, cdk2, or cdc2 in the coronary arterial endothelium. PCNA, c-myb, cdk2, or cdc2 expression of PCNA, cdc2, and cdk2 mRNA was examined.3,13 Briefly, RT solution with 3′ primer was applied to each slide, which was then incubated at 42°C for 15 minutes. These sections were then incubated with PCR solution including 3′ and 5′ primers with the following parameters: 94°C, 1 minute; 55°C, 2 minutes; and 72°C, 1 minute; 35 cycles.

For detecting HVJ in primate systemic organs, we performed RT-PCR with the primers for HVJ F and HN protein.14,15 RNA was extracted from primate cardiac allografts and systemic recipient organs removed at day 28 from HVJ-AVE liposome complex-transfected allograft recipients. RNA derived from organs was amplified using RT-PCR (35 cycles) and compared with positive (using RNA from noninactivated HVJ and recipient liver) and negative controls (primers without RNA). β-Actin was used as an internal control.6

To prove the specific effect of E2F decoy, we performed a gel mobility shift assay. Briefly, nuclear extract was prepared from primate cardiac allografts. E2F decoy phosphorothioate ODN primer was 32P-labeled and incubated for 30 minutes and then loaded onto a 4% polyacrylamide gel. The gels were subjected to electrophoresis and dried.6

All quantitated data were analyzed as mean±SD and compared among the groups using Scheffe’s ANOVA.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

### Results

#### Murine Models

**Histological Findings of the Graft Arteries**

All isografts and allografts kept beating throughout the observation period. Coronary arteries of native hearts and isografts did not develop intimal thickening during this study. At 28 days after transplantation, heavy neointimal thickening was observed in the coronary arteries of untreated allograft recipients. In recipients treated with antisense cdk2 kinase ODN or E2F decoy, arterial intima was mildly thickened. These values did not differ significantly between the antisense and decoy treatment groups; however, they were significantly less than values in the nontreated group. In the E2F decoy recipients 56 days after transplantation, arterial intimal thickening was dramatically prevented. Intimal thickening scores in allografts treated with E2F decoy did not progress from day 28 to day 56. There was a significant difference in the degree of intimal thickening between E2F decoy, antisense cdk2 kinase ODN, and nontreated allografts at day 56. Cell infiltration in myocardium did not differ among the groups at days 28 and 56 (Table 1, Figure 1).

**Cell-Cycle Regulatory Gene Expression on Graft Arteries**

Isografts did not express PCNA, c-myb, cdk2, or cdc2 in the coronary arterial endothelium. PCNA, c-myb, cdk2, or cdc2 expression of PCNA, cdc2, and cdk2 mRNA was examined.3,13 Briefly, RT solution with 3′ primer was applied to each slide, which was then incubated at 42°C for 15 minutes. These sections were then incubated with PCR solution including 3′ and 5′ primers with the following parameters: 94°C, 1 minute; 55°C, 2 minutes; and 72°C, 1 minute; 35 cycles.

For detecting HVJ in primate systemic organs, we performed RT-PCR with the primers for HVJ F and HN protein.14,15 RNA was extracted from primate cardiac allografts and systemic recipient organs removed at day 28 from HVJ-AVE liposome complex-transfected allograft recipients. RNA derived from organs was amplified using RT-PCR (35 cycles) and compared with positive (using RNA from noninactivated HVJ and recipient liver) and negative controls (primers without RNA). β-Actin was used as an internal control.6

To prove the specific effect of E2F decoy, we performed a gel mobility shift assay. Briefly, nuclear extract was prepared from primate cardiac allografts. E2F decoy phosphorothioate ODN primer was 32P-labeled and incubated for 30 minutes and then loaded onto a 4% polyacrylamide gel. The gels were subjected to electrophoresis and dried.6

All quantitated data were analyzed as mean±SD and compared among the groups using Scheffe’s ANOVA.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

### Results

#### Murine Models

**Histological Findings of the Graft Arteries**

All isografts and allografts kept beating throughout the observation period. Coronary arteries of native hearts and isografts did not develop intimal thickening during this study. At 28 days after transplantation, heavy neointimal thickening was observed in the coronary arteries of untreated allograft recipients. In recipients treated with antisense cdk2 kinase ODN or E2F decoy, arterial intima was mildly thickened. These values did not differ significantly between the antisense and decoy treatment groups; however, they were significantly less than values in the nontreated group. In the E2F decoy recipients 56 days after transplantation, arterial intimal thickening was dramatically prevented. Intimal thickening scores in allografts treated with E2F decoy did not progress from day 28 to day 56. There was a significant difference in the degree of intimal thickening between E2F decoy, antisense cdk2 kinase ODN, and nontreated allografts at day 56. Cell infiltration in myocardium did not differ among the groups at days 28 and 56 (Table 1, Figure 1).

**Cell-Cycle Regulatory Gene Expression on Graft Arteries**

Isografts did not express PCNA, c-myb, cdk2, or cdc2 in the coronary arterial endothelium. PCNA, c-myb, cdk2, or cdc2 expression of PCNA, cdc2, and cdk2 mRNA was examined.3,13 Briefly, RT solution with 3′ primer was applied to each slide, which was then incubated at 42°C for 15 minutes. These sections were then incubated with PCR solution including 3′ and 5′ primers with the following parameters: 94°C, 1 minute; 55°C, 2 minutes; and 72°C, 1 minute; 35 cycles.

For detecting HVJ in primate systemic organs, we performed RT-PCR with the primers for HVJ F and HN protein.14,15 RNA was extracted from primate cardiac allografts and systemic recipient organs removed at day 28 from HVJ-AVE liposome complex-transfected allograft recipients. RNA derived from organs was amplified using RT-PCR (35 cycles) and compared with positive (using RNA from noninactivated HVJ and recipient liver) and negative controls (primers without RNA). β-Actin was used as an internal control.6

To prove the specific effect of E2F decoy, we performed a gel mobility shift assay. Briefly, nuclear extract was prepared from primate cardiac allografts. E2F decoy phosphorothioate ODN primer was 32P-labeled and incubated for 30 minutes and then loaded onto a 4% polyacrylamide gel. The gels were subjected to electrophoresis and dried.6

All quantitated data were analyzed as mean±SD and compared among the groups using Scheffe’s ANOVA.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.
were expressed diffusely and strongly in the thickened allograft arterial intima from untreated recipients, whereas treatment with antisense cdk2 kinase ODN or E2F decoy resulted in limited expression in the endothelial cells of the mildly thickened allograft intima. Expression of PCNA, c-myc, cdk2, and cdc2 differed significantly between the groups at day 56 (Table 1, Figure 2).

Primate Models

Histological Findings of the Graft Arteries

In this primate model, all allografts kept beating throughout the observation period. Coronary arteries of native hearts did not show intimal thickening in this study. Although heavy intimal thickening developed in the nontransfected or mismatch decoy-transfected allografts, arterial intima were minimally thickened on day 28 in the grafts transfected with E2F decoy. The intimal thickening of E2F decoy–transfected allografts was significantly less than that of the other 2 groups. Immunohistochemically, PCNA, cdc2, c-myc, and c-myb were diffusely and strongly expressed in the thickened intima of the allograft arteries with no decoy or mismatch decoy transfection, whereas E2F decoy treatment suppressed their expression. SMemb (which is expressed by phenotypically modulated SMCs) was also enhanced in the thickened intima of the allograft arteries with no decoy or mismatch decoy transfection, whereas E2F decoy suppressed SMemb expression. Using in situ RT-PCR, transcription of cell-cycle regulatory genes cdc2 and cdk2 was seen to be strongly and diffusely enhanced in the thickened intima of the no decoy–transfected or mismatch decoy–transfected allografts, whereas E2F decoy treatment suppressed the expression of these mRNA. Expression scores of these 2 groups were significantly higher than those of the E2F decoy–transfected group (Table 2, Figure 3).

Localization and Kinetics of FITC-Labeled Phosphorothioate ODN

We transferred FITC decoy into primate allografts using the HVJ-AVE–liposome method to confirm the gene transfection. Transfection of FITC decoy resulted in widespread distribution of fluorescence in medial vascular SMCs and

---

**Table 2. Pathological Findings of Primate Allografts**

<table>
<thead>
<tr>
<th>Group</th>
<th>Grafts, No.</th>
<th>Arteries, No.</th>
<th>Luminal Occlusion, %</th>
<th>SMemb</th>
<th>PCNA</th>
<th>cdc2</th>
<th>c-myc</th>
<th>c-myb</th>
<th>In Situ RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No deco</td>
<td>4</td>
<td>111</td>
<td>35.7±31.1*</td>
<td>1.0±0.8*</td>
<td>0.9±1.2*</td>
<td>1.0±0.8*</td>
<td>1.2±0.9*</td>
<td>1.2±0.9*</td>
<td>1.7±1.0*</td>
</tr>
<tr>
<td>Mismatch deco</td>
<td>5</td>
<td>128</td>
<td>28.8±25.8*</td>
<td>0.8±0.7*</td>
<td>0.7±1.0</td>
<td>0.8±0.7*</td>
<td>1.1±1.0*</td>
<td>1.1±1.0*</td>
<td>1.8±0.7*</td>
</tr>
<tr>
<td>E2F deco</td>
<td>5</td>
<td>160</td>
<td>16.9±21.0</td>
<td>0.5±0.7</td>
<td>0.4±0.8</td>
<td>0.4±0.7</td>
<td>0.4±0.8</td>
<td>0.4±0.8</td>
<td>1.0±1.0</td>
</tr>
<tr>
<td>Native hearts</td>
<td>2</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. Scoring of the intensity was as follows: 0, no visible staining; 1, few cells with faint staining; 2, moderate staining; and 3, intense diffuse staining.

*P<0.05 vs E2F decoy group.
myocardial cells of primate allografts harvested at day 28; native hearts revealed no specific fluorescence (Figure 4).

**RT-PCR for Detecting Disseminated HVJ**
To confirm the lack of infection of primate systemic organs by UV-irradiated HVJ after ex vivo transfection, we analyzed recipient organs for detection of HVJ mRNA using RT-PCR. It is reported that UV irradiation of HVJ (198 mJ/cm²) greatly reduces infectivity, whereas the fusion activity was still intact. However, it remains to be elucidated whether liposomes fused with UV-irradiated HVJ cause dissemination of intact HVJ in primates. RT-PCR studies revealed that HVJ F protein mRNA in the organs of primates that received HVJ-transfected allografts was not amplified; a single band corresponding to the transcription of these proteins is seen in noninactivated HVJ (Figure 5).

**Gel Mobility Shift Assay**
Gel mobility shift assay analysis documented that increased E2F binding activity was observed in no transfected allografts. This enhanced E2F binding was abolished by E2F decoy; however, the effect was not observed in mismatch decoy (Figure 6).

**Discussion**

**Cell-Cycle Regulatory Genes Are Critical for Development of Graft Arteriopathy**
Graft arteriopathy can be detected in many cardiac transplant recipients treated with conservative immunosuppressants. This coronary disease is one of the most discouraging aspects of cardiac transplantation, characterized by diffuse intimal thickening comprised of proliferative SMCs. Little is known about the pathogenesis of neointimal formation; however, an immune-mediated process is the primary cause of proliferation of SMCs. Because inflammatory cell emigration involves expression of multiple adhesion molecules and other factors by the endothelium, it seems unlikely that selective inhibition of a particular factor would prevent lesion formation completely. It has become clear that cell growth is dependent on the coordinated actions of cell-cycle regulatory genes. Because expression of multiple cell-cycle regulatory genes, such as cdc2, PCNA, and c-myc, was enhanced in the thickened intima of allograft arteries, it can be deduced that E2F must play an important role in the proliferation of vascular SMCs in the rejected allografts.

**E2F Decoy Prevents Neointimal Formation**
We hypothesized that graft arteriopathy after cardiac transplantation could be prevented by blockade of cell-cycle regulatory genes. Several studies indicate that modulation of cell-cycle regulatory genes suppresses SMC proliferation or neointimal formation using several methods targeting retinoblastoma gene products and cyclin inhibitors. These are promising methods to attenuate development of intimal thickening caused by SMC proliferation; however, multiple factors are involved in arteriopathy. Actually, we showed antisense cdk2 kinase ODN treatment prevents neointimal formation with limited effects in this murine model. This result indicates that inhibition of a single cell-cycle regulatory gene is insufficient to prevent vascular SMC proliferation. Thus, we
focused on a transcription factor E2F, which regulates multiple cell-cycle regulatory genes, including PCNA, c-myc, c-myb, cdc2, and cdk2. We used a unique molecular strategy: a synthetic double-stranded DNA with high affinity for a target transcription factor is introduced into target cells as a decoy cis element to bind the transcription factors and alter gene transcription. E2F decoy transfer into the allografts specifically abolished E2F activity and inhibited intimal hyperplasia. In addition, it was more effective than antisense ODN, because it blocked multiple transcriptional factors that bind to the same cis element. Although there are at least several members of the E2F family, this strategy using E2F decoy inhibits all E2F members, because the decoy competitively blocks binding to the cis element. It is noteworthy that the percentage of intimal thickening in murine allografts treated with E2F decoy did not differ between days 28 and 56, indicating that E2F decoy treatment resulted in a plateau curve of intimal thickening development in this study.

Gene Transfer: Clinical Utility for Preventing Graft Arteriopathy

Recently, ex vivo E2F decoy transfection without vectors in vascular grafts suppressed neointimal hyperplasia after cardiac bypass surgery in humans. The results demonstrated that the strategy is clinically promising. However, the studies used the high-pressure method without high-pressure support in vivo. It is impossible to use the high-pressure method in heart allografts, because it would destroy the graft structures of vessels and muscles. In this study, we demonstrated that ex vivo E2F decoy transfection was achieved by the HVJ-AVE–liposome method with high-pressure support. Previous approaches that used ODN as a therapeutic agent in vivo were limited by its short half-life and nonspecific toxicity at high doses and the inefficiency of cellular uptake. Previous approaches that used ODN as a therapeutic agent in vivo were limited by its short half-life and nonspecific toxicity at high doses and the inefficiency of cellular uptake. Therefore, the HVJ-AVE–liposome method increases the efficiency of cellular uptake of ODN without significant side effects. We revealed that this ex vivo decoy transfection into cardiac allografts achieved significant cellular uptake, because FITC expression would be eliminated when the decoy is fragment ed. We also revealed that RT-PCR studies indicated that an intraluminal ex vivo administration of E2F decoy into primate cardiac allografts is unlikely to cause harmful side effects in recipients, because no viral dissemination could be detected. This strategy therefore offers advantages over other methodologies, such as in vivo injection of genes using adenovirus vector, in which viral dissemination is revealed.

Therefore, the efficacy and safety of HVJ-AVE–liposome ex vivo transfection into the transplant organs of this model suggest that clinical trials using this strategy could be feasible. A single intraluminal ex vivo application of HVJ-AVE liposome yields sustained ODN stability and prevents neointimal formation in graft organs and, thus, is a particularly attractive approach in cases of graft arteriopathy after cardiac transplantation.

In this study, intraluminal ex vivo delivery of ODN using our method is clinically feasible; therefore, prevention of graft arteriopathy after cardiac transplantation by this gene therapy is promising.

Acknowledgments

This study was supported by a grant-in-aid from the Ministry of Education, Science and Culture; Research Grant for Immunology, Allergy and Organ Transplant, Ministry of Health and Welfare; Japan Heart Foundation Research Grant; and grant-in-aid from the Kanae Foundation for Life & Socio-Medical Science. We would like to thank Midori Okie and Rie Shiohara for excellent technical assistance.

References


Gene Therapy for Attenuating Cardiac Allograft Arteriopathy Using Ex Vivo E2F Decoy Transfection by HVJ-AVE–Liposome Method in Mice and Nonhuman Primates

Motohiro Kawauchi, Jun-ichi Suzuki, Ryuichi Morishita, Yuko Wada, Atushi Izawa, Naruya Tomita, Jun Amano, Yasufumi Kaneda, Toshio Ogihara, Shinichi Takamoto and Mitsuaki Isobe

_Circ Res._ 2000;87:1063-1068
doi: 10.1161/01.RES.87.11.1063
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/11/1063

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2000/11/15/87.11.1063.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ 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 Research_ is online at:
http://circres.ahajournals.org//subscriptions/