Human Cytomegalovirus Causes Endothelial Injury Through the Ataxia Telangiectasia Mutant and p53 DNA Damage Signaling Pathways


Abstract—Atherosclerosis is the leading cause of death in the United States, and human cytomegalovirus (HCMV), a member of the herpes virus family, may play a role in the development of the disease. We previously showed that HCMV regulated endothelial apoptosis. In this study, we investigated the induction of apoptosis and signal transduction pathways regulating this process in HCMV-infected endothelial cells. As observed previously, HCMV induced a typical cytopathic effect in human aortic endothelial cells (HAECs), ie, the formation of single nucleated or multinucleated giant cells. Although infected HAECs were resistant to apoptosis at earlier stages of infection, they became apoptotic with prolonged infection as demonstrated by positive staining using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). This apoptotic process was mediated by the caspase-dependent mitochondrial apoptotic pathway as indicated by increased expression and cleavage of caspases 3 and 9 as well as increased expressions of pro-apoptotic molecules Bax and Bak. Blocking caspases 3 or 9 significantly inhibited the HCMV-induced apoptosis. Further exploration of the upstream pathway demonstrated upregulation of the tumor suppressor p53 gene and activation of the ataxia telangiectasia mutant (ATM) pathway in the infected cells. Blocking p53 inhibited HCMV-stimulated Bax and Bak expression as well as caspase-3 activation and blocking the ATM pathway inhibited HCMV-stimulated p53 activation. Although early infection may render cells anti-apoptotic, prolonged infection, however, induced endothelial apoptosis through ATM and p53-dependent activation of the mitochondrial death pathway. This proapoptotic effect may be relevant to endothelial dysfunction and HCMV-associated vascular diseases. (Circ Res. 2004;94:1310-1317.)

Key Words: cytomegalovirus ■ endothelium ■ ATM ■ p53 ■ apoptosis

Human cytomegalovirus (HCMV) is a member of the Herpesviridae family and infects 50% to 90% of adults in most populations. An increasing body of evidence suggests that HCMV infection may play a role in vascular diseases, such as atherosclerosis and thrombosis, and allograft rejection. Furthermore, HCMV infection has been shown to promote angiogenesis in mice. However, the mechanisms of the pathogenesis are not clear.

HCMV infects many different tissues and organ systems. Endothelium is unique in many ways mediating HCMV-induced pathogenesis. Endothelium is a critical component within the circulatory compartment, which provides a protective barrier and regulates vascular functions in defense against HCMV invasion. Damage to macrovascular and microvascular endothelium is an important event in many forms of vascular injuries. HCMV has an established tropism in a variety of endothelial cells. Establishment of HCMV propagation within endothelial cells can potentially cause endothelial dysfunction. HCMV infection may initiate endothelial inflammation and vasculitis, which form part of the atherosclerotic or restenotic processes. HCMV infection disturbs endothelial integrity by increased expressions of endothelial surface adhesion molecules that are responsible for leukocyte migration and adhesion to vessel wall. Moreover, HCMV infection is capable of changing endothelium from an anticoagulant to a procoagulant status. Furthermore, endothelium is a reservoir for HCMV latency development. Reactivation of the latent HCMV in these host endothelial cells can immediately cause endothelial damage in large and small vessels—an event that preludes to many forms of vascular injuries. Therefore, understanding the...
molecular events during active and latent infections of endothelium is critical to the understanding of HCMV-induced vascular diseases.

In previous studies, we and others have shown that at earlier stages of HCMV infection (typically the first 72 to 96 hours postinfection, which include the classically defined immediate early, early, and late infection), infected endothelial cells are resistant to apoptotic stimulation such as serum starvation or ultraviolet-induced DNA damage. Inhibition of p53 function either by cytoplasmic sequestration or by direct inhibition on p53 transactivation has been suggested as a responsible mechanism. However, this process of acute infection may not accurately reflect the chronic in vivo pathogenesis of vascular disease, which generally develops over extended periods of time. In the present study, we have extended our investigation to explore the molecular changes in endothelial cells after prolonged HCMV infection. We observed that prolonged HCMV infection induced endothelial apoptosis. We found that caspase-3, caspase-9, proapoptotic Bax, and Bak were induced in HCMV-infected cells. Furthermore, the induction of apoptosis involves ataxia telangiectasia mutant (ATM) -dependent and p53-dependent activation of the mitochondrial death pathway.

**Methods**

**p53 siRNA, Kinase Inhibitor, and Antibodies**

p53 siRNA was purchased from Ambion (Austin, Tex). ATM inhibitor caffeine was purchased from Sigma. Caspase inhibitors (caspase-3 inhibitor II, Z-DEVD-FMK; caspase-8 inhibitor II, Z-IETD-FMK; caspase-9 inhibitor I, Z-LEHD-FMK; the negative control Z-FA-FMK) were purchased from EMD Biosciences (San Diego, Calif). For Western blot analysis and immunofluorescence staining, monoclonal and polyclonal antibodies from Cell Signaling (Beverly, Mass) and Santa Cruz Biotechnology (Santa Cruz, Calif) were used.

**Cell Culture**

Primary human aortic endothelial cells (HAECs) from Cell Applications (San Diego, Calif) were grown in K12 medium, containing 20% fetal bovine serum, penicillin (100 U/mL), streptomycin (10 μg/mL), sodium pyruvate (1 mmol/L), L-glutamine (4 mmol/L), and heparin (30 μg/mL) and supplemented with endothelial cell growth factor (100 μg/mL). Cells cultured up to 5 passages were used in experiments. In all cases, floating and attached cells in each sample were combined for processing at the end of incubation.

**Infection of HAECs With HCMV**

Three strains of HCMV (AD169 [ATCC VR-538], Towne [ATCC VR-977], and VHL/E [from W.J.W.]) were used in the study. The virus was propagated as previously described. HCMV-infected cells were harvested, and the cell-associated virus was released from cells by freeze/thaw cycle. Subconfluent HAEC monolayers were infected with HCMV at multiplicity of infection (MOI) 1 and incubated at 37°C for 1 hour for virus adsorption. The monolayers were then washed 3 times with Dulbecco phosphate-buffered saline (PBS), fresh complete medium was added, and cells were cultured at 37°C in a CO2 incubator. Supernatant and cell fractions were harvested at various postinfection (pi) times.

**p53 siRNAs and Endothelial Cell Transfection**

Silencing of p53 gene expression in primary aortic endothelial cells was achieved using the siRNA technique. Transfection of HAEC was performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instruction.

**Western Blot Analysis**

HAECs were collected from mock-infected and HCMV-infected cultures and washed with ice-cold PBS. Cells were lysed in protein lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerol phosphate, 1 mmol/L Na3VO4, 10 μmol/L of each protease inhibitors [aprotinin, leupeptin and pepstatin], and 1 mmol/L phenylmethylsulfonyl fluoride) for 1 hour on ice. Protein concentration was measured by the Bradford method (Bio-Rad). Fifteen μg of protein per lane was separated by 10% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The membrane was blocked in 5% nonfat powdered milk in TBST (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.05% Tween 20). The membrane was incubated with the primary antibody in 2% powdered milk in TBST, washed extensively with TBST, and then incubated with secondary antirabbit or antionmouse horseradish peroxidase-labeled antibody. Bands were visualized with ECL (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instruction.

**Immunofluorescent Staining and Microscopy**

For immunofluorescence assays, cells were grown on glass coverslips and infected. After infection, the coverslips were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.2% Triton X-100 for 5 minutes. The coverslips were blocked with 1% bovine serum albumin, incubated with the primary antibody, washed extensively with PBS, and then incubated with secondary antirabbit or antionmouse fluorescein isothiocyanate (FITC) or Texas Red-labeled antibody. One percent bovine serum albumin in PBS was used for blocking nonspecific binding sites and for dilution of primary and secondary antibodies. The DNA dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was added at a concentration of 0.1 μg/mL and incubated for 15 minutes to counterstain double-stranded DNA in nuclei. The slides were examined with a Leica DMLS epifluorescence microscope equipped with a Leica DC 100 digital camera and the data were analyzed with Image-Pro Plus V4.5 software (Media Cybernetics, Inc).

**Apoptosis TUNEL Assay**

Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) using in situ cell death detection kit (Roche Applied Science, Indianapolis, Ind) according to the manufacturer’s instruction. Briefly, infected and mock-infected cells were harvested, washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes, and permeabilized with 0.2% Triton X-100 for 5 minutes. The fixed cells were incubated with TUNEL reaction mixture containing the terminal deoxynucleotidyl transferase at 37°C for 1 hour. Double-stranded DNA in nuclei was counterstained after TUNEL staining with DAPI (0.1 μg/mL). The cells were air-dried, and coverslips were placed on a drop of antifade solution and sealed to slide with mounting solution. Images of nuclear fluorescence were obtained by fluorescence microscopy as described.

**Results**

**Cytopathic Effect on Endothelial Cells at Prolonged HCMV Infection**

To investigate whether HCMV infection changes endothelial cell viability/mortality, we infected HAEC with the laboratory-adapted virus strains AD169, Towne, and VHL/E, a clinical isolate whose natural endothelial cytopathogenicity has been preserved by propagation in endothelial cells. Only VHL/E strain demonstrated a high rate of infection at the same level of MOI and clearly showed the typical pathogenicity phenotype. We therefore focused on the VHL/E strain.
for the rest of this study. At earlier stage of infection (days 1 to 5 pi), the infected cells remained healthy and viable (data not shown). However, the cell viability declined in infected cells at later stage of infection (after 5 days pi) compared with mock-infected negative controls (Figure 1A). We next examined whether the HCMV-induced endothelial morphological changes were caused by apoptosis. Internucleosomal fragmentation of cellular DNA is a hallmark of apoptosis. Thus, we evaluated the effects of HCMV infection on the fragmentation of chromosomal DNA by incorporation of fluorescein-dUTP into the 3'-OH of nicked chromosomal DNA (TUNEL analysis). Microscopic analysis of fluorescein-dUTP-labeled DNA showed that infecting HAEcs with HCMV induced apoptosis and the apoptosis was specifically confined in the infected cells (Figure 1B). Kinetic studies showed that apoptotic changes started after 5 days pi and increased with time, indicating that prolonged HCMV infection induced endothelial cell apoptosis (Figure 1C and 1D).

**Activation of the Caspase Apoptosis Pathway by HCMV Infection**

Apoptosis is triggered by caspase-dependent and caspase-independent pathways. In caspase-dependent pathway, apoptotic signals from membrane-associated receptors and mitochondrial sensors converge on a common pathway with the release of cytochrome c from mitochondria and activation of the caspases. Caspase-3 is a terminal caspase effector involved in apoptosis by proteolytically cleaving essential cellular proteins. To investigate whether caspase-3 plays a role in HCMV-induced endothelial apoptosis, we measured caspase-3 expression and activation in HCMV infected cells. As shown in Figure 2A, VHL/E strain-infected cells showed most dramatic changes in expression and activation of caspase-3. Time course showed that caspase-3 (32-kDa) expression noticeably increased 3 days after HCMV infection and was cleaved into 17-kDa to 19-kDa molecules indicating caspase-3 activation. The activation of caspase-3 was specif-
ically in the infected cells as shown in the immunofluorescence stain (Figure 2C). These results indicate that HCMV-induced apoptosis was mediated, at least in part, by caspase-3 activation.

Caspase-3 can be activated through specific proteolytic cleavage by upstream components of different apoptotic pathways. Caspase-9, involved in the mitochondrion-dependent pathway, is the upstream caspase that activates caspase-3. To explore the role of the mitochondrion-dependent pathway in HCMV-triggered apoptosis, we measured the expression and cleavage of caspase-9. As shown in Figure 2A, compared with mock-infected cells, the HCMV-infected endothelial cells had increased expression and cleavage of caspase-9, particularly in VHL/E strain-infected cells. Again, the activation of caspase-9 was specifically in the infected cells as shown in the immunofluorescence stain (Figure 2D). Therefore, we concluded that caspase-9 activation was associated with and likely essential for efficient apoptotic response to HCMV infection. We further showed increased expression and activation of caspase-8 in the infected cells (Figure 2E) indicating that the receptor-mediated apoptosis pathway was also activated.

To further examine whether the HCMV-induced activation of caspases was essential for HCMV-induced apoptosis, we blocked caspase activity with specific inhibitors (caspase-3 inhibitor II, Z-DEVD-FMK for caspase-3; Z-LEHD-FMK for caspase-9; Z-IETD-FMK for caspase-8; and Z-FA-FMK as negative control). As shown in Figure 2F, HCMV-induced apoptosis was suppressed by all 3 caspase inhibitors (ANOVA $F=84.33$, $P<0.001$). The inhibiting effect by caspase-3 or 9 inhibitors was greater than that by caspase-8 ($P=0.024$). These findings...
confirm that caspase activation mediated by either receptor or mitochondrial pathways plays a central role in HCMV-induced apoptosis (Figure 2F).

Expression of Bcl Family Molecules Bax and Bak in HCMV-Infected Endothelial Cells
Activation of caspases is modulated by several mechanisms. Members of the Bcl-2 family, which include antiapoptotic Bcl-2 and Bcl-XL, and the proapoptotic Bax and Bak control the release of cytochrome c from mitochondria and the activation of the caspase pathway. The upregulation of Bax and Bak expression and downregulation of Bcl-2 have been demonstrated during apoptosis. We therefore examined the expression of Bak and Bax in the HCMV-infected endothelial cells. As shown in Figure 3A, the expression of Bax and Bak was low in mock-infected cells. However, there was a dramatic induction in Bax expression by HCMV infection, particularly in VHL/E strain-infected cells. The increase in the expression of both Bax and Bak started at 1 day pi and sustained after 9 days pi (Figure 3B). This increased expression of Bax and Bak was confined to infected cells as indicated by immunofluorescence stains (Figure 3C and 3D). These results indicate the activation of pro-apoptotic Bax and Bak in HCMV-infected endothelial cells and further demonstrate the involvement of the mitochondrial apoptosis pathway in HCMV-induced endothelial apoptosis.

Activation of the p53 Pathway in HCMV-Infected Endothelial Cells
An important regulator of apoptosis is p53. p53 triggers apoptosis in response to a variety of stress stimuli and induces apoptosis by targeting gene regulation and transcription-independent signaling. Bax and Bak are p53 targets and are upregulated in a number of systems during p53-mediated apoptosis and are directly involved in mitochondrial apoptosis. We therefore examined whether p53 was involved in the HCMV-induced endothelial apoptosis. We first tested p53 activation in HCMV-infected cells. Serine residue 15 (ser15) is an important site for p53 DNA binding and interaction with other transcriptional factors, whereas serine 20 (ser20) is a site that binds to MDM2 when dephosphorylated and dissociates with MDM2 when phosphorylated. As shown in Figure 4A and 4B, the phosphorylation of ser15 was increased in VHL/E-infected cells indicating the activation of p53. The phosphorylation of ser20 was also increased, suggesting the activation and stabilization of p53. Furthermore, HCMV-infected endothelial cells had higher p53 levels at the late stage of infection. Whether this elevation in p53 is caused by increased expression or decreased degradation requires further investigation. The p53 appeared to be specifically activated in HCMV-infected cells as indicated by immunofluorescence stains (Figure 4C and 4D). These findings suggest that p53 can be activated by HCMV infection in endothelial cells.

Involvement of the p53 Pathway in HCMV-Induced Endothelial Apoptosis
To examine whether this p53 activation was involved in HCMV-induced apoptosis, we blocked p53 expression using p53-specific siRNA. As shown in Figure 5, HCMV-induced p53 expression was suppressed in the cells transfected with p53 siRNA in a dose-dependent manner. At the same time, the expressions of Bax and Bak, 2 direct targets of p53, were also reduced with p53 siRNA. Moreover, HCMV-induced expression and cleavage of caspase-3 were inhibited by p53 siRNA. These findings indicate that p53 is directly involved in HCMV-induced endothelial apoptosis.

Role of ATM Kinase in HCMV-Induced p53 Phosphorylation
Phosphorylations of p53 on ser15 and ser20 are regulated, in part, by DNA damage-dependent kinases ATM and Chk2. To investigate the upstream signals of HCMV-induced p53 phosphorylation and activation, we measured the activation
of Chk2/ATM pathway. As shown in Figure 6A, the expression of ATM and phosphorylation of Chk2 were increased in HCMV-infected cells indicating the activation of the ATM pathway. To further investigate the involvement of this pathway in HCMV-induced p53 activation, we treated HCMV-infected cells with caffeine—an inhibitor of ATM.27,28 We found that caffeine limited HCMV-induced Chk2 phosphorylation and p53 phosphorylation in a dose-dependent manner (Figure 6B). Thus, the ATM pathway, at least partially, contributes to HCMV-induced p53 activation.

Discussion

HCMV is a widespread opportunistic pathogen that causes acute, latent, and chronic infections. Although the primary infection may be asymptomatic in immunocompetent individuals, the virus can cause a wide variety of severe diseases in immunocompromised hosts. Involvement of HCMV infection has been discovered in atherosclerosis,1–4 thrombosis,5,6 allograft rejection,7,8 and restenosis.9,10 However, the mechanisms of the pathogenesis are not clear. Earlier, we and others showed that HCMV-infected cells were resistant to apoptosis at the earlier stage of infection.23–25 At this stage, the actively proliferating HCMV may increase resistance of endothelial cells to apoptosis, which provides a period long enough for HCMV to use the cellular reproductive machinery for virus propagation. The mechanism of resistance to apoptosis was suggested to be cytoplasmic sequestration of p53.23 We have shown that HCMV can inhibit p53 nuclear localization signal function.24,25 In the present study, we show that HCMV can induce cytopathic effects and apoptotic changes in endothelial cells after a prolonged period of infection (>5 days). This cytopathic effect may occur in vivo.
during chronic infection or reactivation, which leads to endothelial dysfunction and contributes to the pathogenesis of vascular diseases such as atherosclerosis. The biphasic effect of the HCMV on endothelial apoptotic process was a time- and dose-dependent phenomenon. Although HCMV-infected endothelial cells may be resistant to cell death at the earlier stage, prolonged productive infection will induce cell death. Furthermore, exposure to higher doses of HCMV, ie, by a higher MOI, induces this cytopathic effect earlier in the process (data not shown).

In this study, we further addressed the signal transduction pathways for HCMV-induced cell death as proposed in Figure 7. The activation of caspase-3 in HCMV-infected cells indicates the involvement of the common caspase-3 apoptotic pathway in the process. Caspase-3 is a terminal caspase and is involved in the execution of cell death via cleavage of critical cellular proteins such as poly (ADP-ribose) polymerase, lamins, and gelsolin. Caspase-3 is activated by a variety of different stimuli, including infection with HIV, adenovirus, Sendai virus, and sindbis virus. Caspase-3 can be activated by extrinsic and intrinsic pathways. In the current study, we have shown that both pathways can be activated by HCMV infection. The activation of caspase-9 by HCMV infection suggests that this could be an intrinsic mitochondrial-mediated process, for which we have further explored the upstream pathways.

We have demonstrated HCMV-induced expression of pro-apoptotic Bcl-2 members Bax and Bak, which are involved in activation of mitochondrial apoptotic pathway. The activation of Bax and Bak may be a complicated process in HCMV-infected cells. At the earlier stage of infection, Bax and Bak expression increased after 1 day of infection, before the activation of the ATM/Chk2 pathway and p53, which started 5 days postinfection. Whether this Bax and Bak upregulation is induced by HCMV invasion or immediate early genes (genotoxic stress) or both is interesting to examine. Even though the Bax and Bak levels increased at earlier stage of infection, this activation did not lead to apoptosis. It suggests that there may be virus-induced antiapoptotic mechanisms that can antagonize Bax and Bak during this earlier infection stage for which further investigation is needed. However, antiapoptotic Bcl-2 may not be involved because phosphorylation and expression of Bcl-2 remain unchanged in the infected cells (data not shown). At the later stage of infection, the activation of Bax and Bak is partially mediated by p53 because it can be inhibited by p53 siRNA. The activation of Bax and Bak at this stage may be related to virus replication-induced genotoxic stress that induces apoptosis.

p53 is considered the guardian of the genome and has a number of biological functions including cell cycle arrest, DNA repair, and apoptosis. Our study indicates that p53 activation may mediate the apoptotic changes in prolonged HCMV infection. Blocking p53 expression by siRNA resulted in reduced expression of Bax and Bak suggests that the HCMV-induced upregulation of Bax and Bak is p53-dependent. Bax and Bak as p53 targets are upregulated in a number of systems during p53-mediated apoptosis and are directly involved in mitochondrial apoptosis. In addition to expression, p53 may also be responsible for direct Bax and Bak activation in the HCMV-infected endothelial cells. However, where the p53-mediated Bax/Bak activation takes place remains to be resolved. As we can see from Figure 4C, there was a large proportion of the activated p53 located in cytoplasm and only a small proportion was in nucleus. Whether this small amount active nuclear p53 would be sufficient or whether p53 could directly trigger the Bax/Bak action in cytoplasm or mitochondria is an interesting question to explore. Indeed, recent studies show that p53 has an extranuclear role in the cytoplasm to directly induce apoptosis.

The nuclear kinase ATM and its related kinase ATR are critically involved in integrating the initial DNA damage signals to cell cycle checkpoints and apoptosis. Activation of ATM/ATR by DNA damage has been widely demonstrated and ATM is the key regulatory molecule in DNA damage signaling and apoptosis. ATM resides predominantly in the nucleus and responds to DNA damage by phosphorylating numerous substrates. p53 is the main target of the ATM pathway, which has been reported to directly phosphorylate p53 on ser15. Our experiments have shown that ATM/Chk2 pathway was activated in HCMV-infected cells and blocking ATM activity inhibited p53 phosphorylation on ser15 and ser20 sites. These findings suggest that ATM pathway may directly mediate HCMV-induced p53 phosphorylation on these 2 sites. We have also shown the phosphorylation and activation of Chk2–a checkpoint kinase. Because Chk2 can directly phosphorylate p53 on ser20, the observed phosphorylation on p53 ser20 may also be mediated by Chk2 kinase. However, further kinase assay may be needed to show whether ATM and Chk2 from HCMV-infected cells can directly phosphorylate p53 in vitro. Because ATM kinase is a key regulator of multiple signaling cascades that respond to DNA damage, the involvement of ATM pathway indicates that HCMV-induced endothelium damage may be caused by virus-induced DNA damage or genotoxic stress.
In summary, we have shown that prolonged HCMV infection can induce endothelial apoptosis. Contrary to the antiapoptotic effect during earlier stage of HCMV infection, the induction of apoptosis after prolonged HCMV infection could be responsible for endothelial dysfunction, damage to endothelial integrity, and ensuing vascular diseases in those with chronic or reactivated latent HCMV infection. The elucidation of the molecular mechanisms leading to activation of intrinsic mitochondrial apoptotic pathway through ATM and p53 regulation will offer potential targets to attenuate the HCMV-induced cytopathic effects and, hence, vascular diseases by HCMV infection.

Acknowledgments
This project is supported by a National Institutes of Health/National Heart, Lung and Blood Institute grant R01-HL071608. X.L.W. is an AHA Established Investigator.

References
Human Cytomegalovirus Causes Endothelial Injury Through the Ataxia Telangiectasia Mutant and p53 DNA Damage Signaling Pathways

Circ Res. 2004;94:1310-1317; originally published online April 22, 2004;
doi: 10.1161/01.RES.0000129180.13992.43

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 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/94/10/1310

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/