Activated Interstitial Myofibroblasts Express Catabolic Enzymes and Mediate Matrix Remodeling in Myxomatous Heart Valves

Elena Rabkin, MD, PhD; Masanori Aikawa, MD, PhD; James R. Stone, MD, PhD; Yoshihiro Fukumoto, MD, PhD; Peter Libby, MD; Frederick J. Schoen, MD, PhD

Background—The mechanisms of extracellular matrix changes accompanying myxomatous valvular degeneration are uncertain.

Methods and Results—To test the hypothesis that valvular interstitial cells mediate extracellular matrix degradation in myxomatous degeneration by excessive secretion of catabolic enzymes, we examined the functional characteristics of valvular interstitial cells in 14 mitral valves removed for myxomatous degeneration from patients with mitral regurgitation and in 11 normal mitral valves obtained at autopsy. Immunohistochemical staining assessed (1) cell phenotype using antibodies to α-actin (microfilaments), vimentin and desmin (intermediate filaments), smooth muscle myosin (SM1), and SMemb (a nonmuscle myosin produced by activated mesenchymal cells) and (2) the expression of proteolytic activity using antibodies to collagenses (matrix metalloproteinase [MMP]-1, MMP-13), gelatinases (MMP-2, MMP-9), cysteine endoproteases (cathepsin S and K), and interleukin-1β, a cytokine that can induce secretion of proteolytic enzymes. Although interstitial cells in normal valves stained positively for vimentin, but not α-actin or desmin, cells in myxomatous valves contained both vimentin and α-actin or desmin (characteristics of myofibroblasts). Moreover, cells in myxomatous valves strongly expressed SMemb, MMPs, cathepsins, and interleukin-1β, which were weakly stained in controls. Nevertheless, interstitial cells in both groups strongly expressed procollagen-I mRNA (in situ hybridization), suggesting preserved ability to synthesize collagen in myxomatous valves.

Conclusions—Interstitial cells in myxomatous valves have features of activated myofibroblasts and express excessive levels of catabolic enzymes, without altered levels of interstitial collagen mRNA. We conclude that valvular interstitial cells regulate matrix degradation and remodeling in myxomatous mitral valve degeneration.

Key Words: mitral valve remodeling metalloproteinases collagen

Myxomatous degeneration is the pathological substrate of mitral valve prolapse, a common cardiac valvular disorder characterized by redundant, floppy leaflets and associated with progressive mitral regurgitation, thromboembolism, infective endocarditis, and sudden death.1,2 Floppy valves likely result from mechanically inadequate extracellular matrix (ECM), particularly collagen, thereby allowing stretching of the leaflets.3 Nevertheless, the mechanisms responsible for the ECM defect and/or reparative processes that bolster the defective ECM are uncertain.

As in other tissues, turnover of the valvular ECM depends on a dynamic balance between synthesis and degradation. In most tissues, degradation of the ECM occurs through the action of matrix metalloproteinases (MMPs) and cysteine endoproteases (cathepsins). In cardiac valves, tight regulation of matrix homeostasis stringently maintains the functional architecture of the normal valve.4 Therefore, we propose that dysregulation of matrix metabolism modulates major features of the abnormalities of collagen and other ECM in heart valves with myxomatous degeneration.

The present study evaluated the role of valvular interstitial cells and catabolic enzymes such as MMPs and cathepsins in the pathogenesis of myxomatous degeneration using valves from patients who required surgery for severe mitral regurgitation, had myxomatous degeneration on pathological examination, and had no other documented condition known to predispose to mitral regurgitation.
TABLE 1. Normal Valves

<table>
<thead>
<tr>
<th>Age, y*</th>
<th>Sex</th>
<th>Cause of Death</th>
<th>Post Mortem Interval, hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>6</td>
</tr>
<tr>
<td>52</td>
<td>F</td>
<td>Carcinoma</td>
<td>20</td>
</tr>
<tr>
<td>77</td>
<td>F</td>
<td>Cerebral aneurysm</td>
<td>4</td>
</tr>
<tr>
<td>78</td>
<td>F</td>
<td>Pseudomembranous colitis</td>
<td>3</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>Subarachnoid hemorrhage</td>
<td>8</td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>Scleroderma</td>
<td>15</td>
</tr>
<tr>
<td>46</td>
<td>F</td>
<td>Sickle cell disease</td>
<td>20</td>
</tr>
<tr>
<td>80</td>
<td>M</td>
<td>Liposarcoma</td>
<td>5</td>
</tr>
<tr>
<td>61</td>
<td>F</td>
<td>COPD</td>
<td>19</td>
</tr>
<tr>
<td>30</td>
<td>F</td>
<td>Leukemia</td>
<td>12</td>
</tr>
<tr>
<td>53</td>
<td>M</td>
<td>Mesothelioma</td>
<td>9</td>
</tr>
</tbody>
</table>

COPD indicates chronic obstructive pulmonary disease.

*Mean age was 54.7±5.3 years.

Methods

Mitrval valves with myxomatous degeneration derived from valve repair (n=13) or replacement surgery (n=1) and normal mitral valves from autopsy (n=11) were obtained according to a protocol approved by the Human Research Committee at the Brigham and Women’s Hospital. Detailed review of clinical charts revealed that none of the patients with myxomatous valve degeneration had documented coronary artery disease or other conditions known to predispose to mitral regurgitation (Tables 1 and 2).

Morphological Characterization

Specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (6 µm) were stained with hematoxylin and eosin for general morphology, Movat pentachrome stain for connective tissue elements (differentially stains collagen, elastin, and proteoglycans),5 and picrosirius red for collagen architecture and evaluation of collagen types I and III.6,7

TABLE 2. Myxomatous Valves

<table>
<thead>
<tr>
<th>Age, y*</th>
<th>Sex</th>
<th>Clinical Diagnosis/Operative Findings</th>
<th>Tissue Examined (leaflet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>F</td>
<td>MR4+, MVP, HT</td>
<td>P</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>68</td>
<td>M</td>
<td>MR4+, MVP, HT, CR</td>
<td>P</td>
</tr>
<tr>
<td>64</td>
<td>M</td>
<td>MR4+, MVP</td>
<td>P</td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>57</td>
<td>M</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>52</td>
<td>M</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>58</td>
<td>M</td>
<td>MR4+, MVP</td>
<td>P</td>
</tr>
<tr>
<td>71</td>
<td>F</td>
<td>MR4+, MVP</td>
<td>P</td>
</tr>
<tr>
<td>57</td>
<td>M</td>
<td>MR4+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>MR3+, MVP, CR</td>
<td>P</td>
</tr>
<tr>
<td>47</td>
<td>F</td>
<td>MR3+, MVP, TR</td>
<td>P</td>
</tr>
<tr>
<td>42</td>
<td>M</td>
<td>MR4+, MVP</td>
<td>P+A</td>
</tr>
</tbody>
</table>

MR indicates mitral regurgitation; MVP, mitral valve prolapse; HT, hypertension; TR, tricuspid regurgitation; AI, aortic insufficiency; CR, chordal rupture; P, posterior; and A, anterior.

*Mean age was 55.4±2.7 years.

TABLE 3. Antibodies Used to Characterize Myofibroblasts and Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Filament System</th>
<th>Antigen reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>Intermediate—mesenchymal</td>
<td>+</td>
</tr>
<tr>
<td>Desmin</td>
<td>Intermediate—muscle</td>
<td>+/-</td>
</tr>
<tr>
<td>α-Actin</td>
<td>Microfilament—actin</td>
<td>+/-</td>
</tr>
<tr>
<td>SM1</td>
<td>Myosin heavy chain</td>
<td>–</td>
</tr>
<tr>
<td>SM2</td>
<td>Myosin heavy chain</td>
<td>–</td>
</tr>
</tbody>
</table>

SMC indicates smooth muscle cells.

Characterization of Myofibroblasts and Detection of Catabolic Enzymes

Myofibroblasts were distinguished by antibody reaction to cytoskeletal filaments (smooth muscle α-actin [microfilaments] and vimentin and desmin [intermediate filaments; DAKO]), and by negative staining to the myosin antibodies SM1 (differentiated smooth muscle cells) and SM2 (mature smooth muscle cells)9,10 (Table 3). Combined use of these antibodies serves as useful markers to distinguish smooth muscle cells from myofibroblast-like cells. A classification system of myofibroblasts is based on immunohistochemical staining of cytoskeletal filaments.11 Myofibroblasts that express vimentin and α-actin are called VA type, those that express vimentin and desmin are called VD type, and those that express vimentin, α-actin, and desmin are called VAD type. Differentiated smooth muscle cells were determined by immunoreaction to SM1 and SM2 antibodies. Inflammatory cells were identified with antibodies to CD68 (macrophages) and CD45 (T cell; DAKO). Mouse monoclonal antibodies against human MMP-1, MMP-2, MMP-9, and MMP-13 (Oncogene Research Products), and rabbit anti-human cathepsin S and cathepsin K were used to determine the expression of collagenolytic and elastolytic enzymes. Expression of interleukin (IL)-1β (a pro-inflammatory cytokine that induces the expression of MMPs) was examined with goat polyclonal anti-human IL-1β (Santa Cruz Biotechnology), and cell activation was demonstrated by a monoclonal antibody to SMemb10 (a nonmuscle myosin also known as MHC-B). Immunohistochemistry was done using the avidin-biotin-peroxidase method after antigen retrieval. Double immunofluorescent staining was done to confirm coexpression of IL-1β and MMP-13 or cathepsin S. Adjacent sections treated with nonimmune IgG served as controls for antibody specificity.

In Situ Hybridization

Nonisotopic in situ hybridization for human procollagen-I mRNA was done according to the manufacturer’s instructions (InnoGenex). Sections were incubated with oligomers as follows: anti-sense: 5'-GCT CCT TCA ATC CAT CCA GAC CAC A-3', 5'-TAA CCA AAG TCA TAA CCA CCG C-3', 5'-CAT AGT CCT TGG GTC TGA G-3'; sense: 5'-TTG TCT GGA TGG ATT GAA GGG ACA GC3', 5'-GGG GTG GTT ATG ACT TTG CTT A-3', 5'-CTC AGA CCC AAC GAG CAC TAT G-3'.

Quantification and Statistical Analysis

Immunohistochemical staining of MMPs and in situ hybridization for procollagen-I mRNA expression were graded as follows: 0, no appreciable staining; 1, weak in <50% of cells; 2, weak in ≥50% of cells or strong in >10% of cells; 3, strong in 10% to 50% of cells; and 4, strong in ≥50% of cells. Semiquantitative analysis was done by E.R. and an observer without the knowledge of the origin of tissue or antibodies. Valve thickness was measured using a linear eyepiece reticle (10 mm, 0.1 mm division) (Fisher Scientific) for each section in the middle portion of each leaflet and was expressed as a mean of 3 measurements. Cell density was...
expressed as mean number of cells per 10 high power fields. Quantitative data were compared in both groups by Mann-Whitney’s U test. Data are presented as mean±SEM. *P*<0.05 was considered significant.

**Results**

**Myxomatous Valves Show Altered Morphology**

Figure 1 compares the histological features of normal and myxomatous valves. Analogous to aortic valves, normal mitral valves had 3 well-defined tissue layers (Figure 1A), each containing cells and characteristic ECM composition and configuration: (1) the fibrosa, which is composed predominantly of collagen fibers arranged parallel to the free edge of the leaflet, densely packed and microscopically crimped; (2) the centrally located spongiosa, which is composed of loosely arranged collagen and proteoglycans; and (3) the atrialis, which is composed of elastic fibers. In contrast, myxomatous valves showed: (1) expansion of the spongiosa by loose, amorphous ECM staining strongly positive for proteoglycans; (2) diminished staining for collagen fibers (yellow); and (3) fragmentation of elastin (black). Picrosirius red staining under polarized light further demonstrated that individual collagen bundles were fragmented, coiled, disrupted, and disoriented in myxomatous leaflets; collagen fibers were almost undetectable in the spongiosa and had lower birefringence than those in normal valves. Myxomatous valves often had a layer of superficial plaque characterized by the accumulation of stellate and spindle-shaped cells, particularly on the ventricular aspect of the leaflet. Owing to both expansion of the spongiosa and plaque formation, myxomatous leaflets were significantly thicker than normal (2.27±0.1 mm versus 0.92±0.08 mm, respectively; *P*<0.0001; Figure 1B).

Inflammatory cells, including macrophages and T-cells, were negligible in myxomatous valves. The cell density in the spongiosa of myxomatous valves was significantly increased (approximately tripled) compared with normal (62.9±5.5 and 21.2±1.3 cells per high power field, respectively; *P*<0.0001; Figure 1C).

**Figure 1.** Morphological features of normal and myxomatous mitral valves. A, Normal mitral valves (left) and valves with myxomatous degeneration (right). Myxomatous valves have an abnormal layered architecture: loose collagen in fibrosa, expanded spongiosa strongly positive for proteoglycans, and disrupted elastin in atrialis (top). Top, Movat pentachrome stain (collagen stains yellow; proteoglycans, blue-green; and elastin, black). Bottom, Picrosirius red staining viewed under polarized light detected disruption and lower birefringence of collagen fibers in myxomatous leaflets. Bar=200 μm. Magnification ×100. B, Quantitative analysis of valve thickness, demonstrating thickening of myxomatous valves. C, Increased density of interstitial cells in myxomatous spongiosa. Bars represent SEM.
Myxomatous Valves Contain Activated Myofibroblasts

Myofibroblast-like cells accumulated in myxomatous leaflets (Figure 2). In normal valves, interstitial cells expressed vimentin (V phenotype), but not α-actin or SM1. Cells stained with α-actin and antibodies to SM1 and SM2 localized in the proximal (near annulus) and middle portions and distributed predominantly in regions adjacent to the atrial aspect of the leaflet. In both groups, smooth muscle cells (detected by both α-actin and SM1) accumulated in the subendothelial layer (middle and bottom). Note that interstitial cells in myxomatous valves showed undetectable levels of SM1 myosin (bottom right). Bar=50 μm. Magnification ×400.

Myxomatous Valves Overexpress Proteolytic Enzymes

Interstitial cells in myxomatous valves expressed higher levels of catabolic enzymes than in normal valves, especially collagenases (MMP-1, MMP-13; Figure 4A). Semi-quantitative analysis showed that levels of MMP-1, MMP-13, MMP-2, and MMP-9 in myxomatous valves (grades 3.1±0.2, 3.3±0.13, 2.07±0.2, and 2.08±0.2, respectively) were significantly higher than those in normal (grades 1.27±0.2, 0.91±0.3, 1.18±0.2, and 1.27±0.1; P=0.0002, P=0.0001, P=0.02, and P=0.03, respectively; Figure 4B). In normal valves, sparse macrophages could be responsible for low levels of proteolytic enzymes.

Normal valves contained no cathepsin S or K or IL-1β (data not shown). In myxomatous, interstitial cells immu-
noreactive to IL-1β, MMP-13 and cathepsin K colocalized with loose collagen and fragmented elastin in areas of myxomatous degeneration (Figure 4C). Immunofluorescent double-labeling for catabolic enzymes and IL-1β also showed coexpression of MMP-13/IL-1β and cathepsin S/IL-1β by interstitial cells in the spongiosa of myxomatous valves (Figure 4D).

Collagen-I mRNA Expression Is Similar in Myxomatous and Normal Valves
In situ hybridization using oligomers specific for human procollagen-I detected mRNA expression by interstitial cells in both normal and myxomatous valves (Figure 5A). Semiquantitative grading of procollagen-I mRNA expression showed no significant difference between normal (3.1±0.3) and myxomatous valves (3.4±0.25; P=NS; Figure 5B).

Discussion
This study showed that myxomatous valves have significant thickening and highly abnormal layered architecture and ECM components and that interstitial cells in myxomatous leaflets (1) exhibited features of activated myofibroblasts, (2) expressed elevated levels of proteolytic enzymes, (3) retained the ability to express interstitial collagen, and (4) significantly increased in number in the spongiosa. These results suggest that dysregulation of matrix metabolism modulates the abnormalities in collagen and other ECM components in this pathological condition.

Valvular Functional Architecture
Heart valves have a complex, layered architecture and highly specialized, functionally adapted cells and ECM. The ECM elements accommodate repetitive changes in shape and dimension throughout the cardiac cycle; interstitial cells synthesize the ECM and mediate its ongoing repair and remodeling, thereby providing durability. Each of the valvular layers is enriched in a specific ECM component and has a distinct function. These features have been demonstrated predominantly in the aortic valves. Although less work has been done to understand structure-function correlations in the normal mitral valve, the general architecture and roles of the layers are likely analogous to those of the aortic valve. In the normal aortic
defective ECM and altered layered architecture. Indeed, a recent study demonstrated dramatic defects in the mechanical properties of myxomatous leaflets and chordae; myxomatous leaflets were more extensible and half as strong as normal valve tissue, which suggested that the abnormal stresses engendered by progressive stretching of enlarged leaflets and inherent weakness are synergistic in valve degeneration, possibly following as well as preceding valve repair.15

**Interstitial Valvular Cells**

The physiological role of valvular cells in the maintenance and remodeling of ECM in normal valves and their dysregulation in disease are poorly understood. The resting interstitial cell population of normal cardiac valves is predominantly fibroblast-like. These cells synthesize collagen, elastin, and proteoglycans. However, smooth muscle cells and myofibroblasts have also been cultured from heart valves.16–18 Myofibroblasts may well represent an intermediate state between fibroblasts and smooth muscle cells. Detection of myofibroblasts is difficult because of their heterogeneity and absence of particular immunohistochemical markers. Characterization of myofibroblasts is based on antibody reaction to 2 filament systems of cells composed of (1) actin (a component of the microfilaments) and (2) vimentin, desmin, and laminin (members of the intermediate filament system).9 In the present study, interstitial cells in normal valves expressed vimentin, but not α-actin or desmin, although other investigators have often observed α-actin–positive cells cultured from cardiac valves and characterized them as myofibroblasts.16–18 In control valve specimens, only smooth muscle cells displayed α-actin and SM1. Interestingly, in myxomatous leaflets, many cells in deep atrialis and spongiosa expressed vimentin, as well as α-actin or desmin but not SM1, suggesting that under pathological conditions interstitial cells may express features of VA or VD phenotypes of myofibroblasts.

The expression of MMPs, cathepsins, and IL-1β in myxomatous lesions and associated superficial plaques suggest activation of valvular cells. Activation of myofibroblasts was confirmed by SMemb, which is predominantly expressed by activated mesenchymal cells.9,10 Stellate myofibroblasts (large cells with long cytoplasmic extensions) were found in loose myxomatous stroma. These cells can be a local source of multiple cytokines and catabolic enzymes that affect cell proliferation, activation, and ECM accumulation. They can be found in restenotic lesions after angioplasty or stenting,19 suggesting similar mechanisms of myofibroblast activation, differentiation, proliferation, and ECM degradation.

**Role and Mechanisms of Matrix Remodelling in Myxomatous Mitral Valve Degeneration**

Members of a family of enzymes that includes interstitial collagenases and gelatinases, MMPs are involved in the matrix breakdown in normal and pathological conditions such as atherosclerosis6,7 and aortic aneurysms.13 Interstitial collagenases mediate the initial step of collagen

---

**Figure 5.** In situ detection of collagen synthesis. A, Procollagen-I mRNA expression by interstitial cells in normal and myxomatous valves detected by in situ hybridization using antisense oligomers specific for human procollagen-I (red reaction product). No signal was detected with sense probes used as negative control. Bar=50 μm. Magnification ×400. B, Semiquantitative analysis for procollagen-I mRNA expression showed no significant difference between normal and myxomatous valves. Bars represent SEM.
Pathogenesis of myxomatous mitral valve. GAG indicates glycosaminoglycans.

degradation by breakdown of the native helix of the fibrillar collagen network (type I is most abundant, comprising ~70% of the total collagen in valves). These fragments then become accessible to the other proteases, such as gelatinases, which further catabolize collagen. Cathepsins S and K are also involved in ECM remodeling, particularly elastin. Cathepsin K, the most potent elastase yet described, also has collagenolytic activity.

Several lines of evidence suggest that resident cells mediate ECM degradation in many degenerative diseases. Stimulation of these cells in some way elicits soluble extracellular messengers that, in turn, induce resident cells to initiate matrix degradation. For example, cardiac catabolic factor, which is derived from porcine heart valves, stimulates collagen and proteoglycan degradation in vitro. It has properties similar to those of catabolin, a cytokine first detected in porcine synovium and considered a form of porcine IL-1. Previous studies have shown that cultured smooth muscle cells exposed to IL-1β release MMP-2, MMP-9, and cathepsins S and K.

The present study indicates that resident valvular cells contribute to matrix destruction and remodeling in myxomatous valves. Interstitial valvular cells were the major source of the significantly increased expression of proteolytic enzymes, such as MMPs, that mediate ECM degradation and were significantly increased in number compared with normal valves, suggesting cell proliferation. Our study also demonstrated the presence of interstitial cells immunoreactive for cathepsins S and K and strongly expressing IL-1β in the myxomatous valves. Moreover, IL-1β co-localized with MMP-13 and cathepsin S. These observations suggest that interstitial cells induced by IL-1β could mediate the expression of catabolic enzymes and modulate the development of the defective ECM by enzymatic breakdown of the connective tissue first described by Caulfield et al in myxomatous valves.

The present study provides new insight into mechanisms by which ongoing and progressive matrix degeneration may contribute to a common clinical pathway. These data clearly establish that interstitial cells express excessive catabolic enzymes in valves with myxomatous degeneration. However, it remains to be determined whether high proteolytic activity and cell activation in the mitral leaflets are causal or whether regurgitation and abnormal mechanical stress induce matrix remodeling as a reactive mechanism. Nevertheless, these observations on diseased human tissues cannot definitely resolve the nature of the primary stimulus to myxomatous degeneration. Our data implicate a progression of cell/tissue level to clinical events, which is summarized schematically in Figure 6. They also suggest a mechanism by which progressive deterioration of myxomatous tissue could occur after otherwise successful valve repair surgery.

Conclusions

Taken together, these results suggest that the altered layered architecture of the leaflet and structural abnormalities of connective tissue components, as well as excessive levels of collagenolytic and elastolytic enzymes expressed by activated interstitial cells, could cause the leaflet functional abnormalities of myxomatous valves in patients with mitral regurgitation.

Acknowledgments

This project was supported in part by National Heart, Lung, and Blood Institute grant PO1 HL-48743 (to P.L.). The authors thank Dr Richard N. Mitchell for critical review of this work, Dr Guo-Ping Shi (University of California, San Francisco) for the anti-cathepsin antibodies, Eugenia Shvartz and Dmitriy Zvagelsky for technical assistance, Karen Williams for editorial expertise, and Claudia Davis for preparing the manuscript.

References


Activated Interstitial Myofibroblasts Express Catabolic Enzymes and Mediate Matrix Remodeling in Myxomatous Heart Valves
Elena Rabkin, Masanori Aikawa, James R. Stone, Yoshihiro Fukumoto, Peter Libby and Frederick J. Schoen

_Circulation_. 2001;104:2525-2532
doi: 10.1161/hc4601.099489

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/104/21/2525

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/