Modulation of Transforming Growth Factor-β Signaling and Extracellular Matrix Production in Myxomatous Mitral Valves by Angiotensin II Receptor Blockers

Arnar Geirsson, MD; Mansher Singh, MD; Rahmat Ali, MD; Hussain Abbas, MD; Wei Li, MD; Juan A. Sanchez, MD; Sabet Hashim, MD; George Tellides, MD, DPhil

Background—Little is known about the pathophysiology of myxomatous degeneration of the mitral valve, the pathological hallmark of mitral valve prolapse, associated with symptomatic mitral regurgitation, heart failure, and death. Excess transforming growth factor (TGF)-β signaling is known to cause mitral valve degeneration and regurgitation in a mouse model of Marfan syndrome. We examined if TGF-β signaling is dysregulated in clinical specimens of sporadic mitral valve prolapse compared with explanted nondiseased mitral valves and we tested the effects of angiotensin II receptor blockers on TGF-β signaling in cultured human mitral valve cells.

Methods and Results—Operative specimens, cultured valve tissues, and cultured valvular interstitial cells were obtained from patients with mitral valve prolapse undergoing mitral valve repair or from organ donors without mitral valve disease. Increased extracellular matrix in diseased valve tissue correlated with an upregulation of TGF-β expression and signaling as evidenced by SMAD2/3 phosphorylation. Both TGF-β ligand and signaling mediators colocalized primarily to valvular interstitial cells suggesting autocrine/paracrine activation. In cultured valve tissue, exogenous TGF-β increased basal extracellular matrix production, whereas serological neutralization of TGF-β inhibited disease-driven extracellular matrix overproduction. TGF-β-induced extracellular matrix production in cultured valvular interstitial cells was dependent on SMAD2/3 and p38 signaling and was inhibited by angiotensin II receptor blockers.

Conclusions—TGF-β has a profibrotic role in the pathogenesis of sporadic mitral valve prolapse. Attenuation of TGF-β signaling by angiotensin II receptor blockers may represent a mechanistically based strategy to modulate the pathological progression of mitral valve prolapse in patients. (Circulation. 2012;126[suppl 1]:S189–S197.)

Key Words: mitral valve ■ molecular biology ■ surgery ■ valves

Sporadic mitral valve prolapse (MVP) is the most common cardiac valvular abnormality in industrialized nations.1 Despite well-characterized histological changes of myxomatous degeneration with accumulation of glycosaminoglycans, collagen fragmentation, disorganized extracellular matrix (ECM), and increased expression of proteolytic enzymes, the molecular pathogenesis is not well defined.2–4 A leading cause of isolated mitral regurgitation, MVP is associated with various complications such as infectious endocarditis, heart failure, atrial fibrillation, stoke, and death.5,6 Most MVP seen in clinical practice is sporadic and of unknown etiology, but an indication of transforming growth factor (TGF)-β involvement derives from both familial and syndromic MVP. X-linked myxomatous valvular dystrophy characterized by multivalve myxomatous degeneration is associated with mutations of filamin A, a ubiquitous phosphoprotein involved in the regulation of various cell signaling molecules, including SMAD2, a primary mediator of TGF-β signaling.7,8 Marfan syndrome is caused by mutation of FBN1 encoding for fibrillin-1, a principal component of extracellular matrix microfibrils that targets latent TGF-β complexes resulting in altered matrix sequestration and dysregulation of TGF-β signaling.9,10 Although MVP is a less common phenotypic feature than aortic root aneurysms, the majority of patients with Marfan have evidence of MVP and many undergo operative treatment for mitral regurgitation in which surgical specimens show evidence of myxomatous degeneration in prolapsed segments as well as predominant fragmentation and disruption of elastin.11 FBN1 mutant mice develop MVP with similar phenotypic features to sporadic MVP that can be rescued by TGF-β neutralizing antibody.12 TGF-β also activates and mediates differentiation of cultured porcine valvular interstitial cells (VICs) to a contractile myofibroblast phenotype evident by α-smooth muscle actin expression and is
required for SMAD2/3-dependent proliferation and wound healing in vitro. These studies suggest that TGF-β may mediate pathological changes that occur in myxomatous mitral valve disease. However, no studies to date have shown an association between sporadic MVP and TGF-β dysregulation in human tissues. The purpose of this study was to examine if TGF-β signaling is dysregulated in human MVP compared with explanted nondiseased mitral valves and to use cultured human VICs to determine the effects of angiotensin II receptor blockers (ARBs) with described TGF-β inhibitory activity on downstream signaling and ECM production.

Methods

Mitrval Valve Tissue

Human tissues were obtained using research protocols approved by the Institutional Review Boards of Yale University, the Veterans Affairs Connecticut Healthcare System, and/or the New England Organ Bank. Specimens were deidentified but the protocol allowed for recording of medications to determine if patients were on ARB before operation and those samples were excluded. Myxomatous mitral valve tissue (hereafter referred to as diseased) was obtained from patients undergoing repair for severe mitral regurgitation with resection of the central scallop of the posterior leaflet. A total of 26 diseased tissue samples was collected. Experienced cardiac surgeons (A.G., S.H., and G.T.) determined the diagnosis of myxomatous mitral valve disease by gross inspection and carefully excluded other mitral valve pathology including stenosis, calcific degeneration, and endocarditis as well as any uncertain diagnosis of myxomatous degeneration. A portion of tissue was submitted to surgical pathology confirming myxomatous degeneration changes in all samples used. A total of 23 nondiseased mitral valves was obtained primarily from organ/tissue donors and also from explanted hearts of transplant recipients without abnormalities suggestive of mitral valve disease by gross inspection and carefully excluded other mitral valve pathology including stenosis, calcific degeneration, and endocarditis as well as any uncertain diagnosis of myxomatous degeneration. A portion of tissue was submitted to surgical pathology confirming myxomatous degeneration changes in all samples used. A total of 23 nondiseased mitral valves was obtained primarily from organ/tissue donors and also from explanted hearts of transplant recipients without abnormalities suggestive of mitral valve disease by gross inspection and carefully excluded other mitral valve pathology including stenosis, calcific degeneration, and endocarditis as well as any uncertain diagnosis of myxomatous degeneration. A portion of tissue was submitted to surgical pathology confirming myxomatous degeneration changes in all samples used. A total of 23 nondiseased mitral valves was obtained primarily from organ/tissue donors and also from explanted hearts of transplant recipients without abnormalities suggestive of mitral valve disease by gross inspection and carefully excluded other mitral valve pathology including stenosis, calcific degeneration, and endocarditis as well as any uncertain diagnosis of myxomatous degeneration. A portion of tissue was submitted to surgical pathology confirming myxomatous degeneration changes in all samples used.

Tissue Culture Experiments

Valve tissue was placed in serum-free M199 medium and “rested” from procurement-associated stress responses for 72 hours before treatment. Cultured valve tissue was treated with carrier or TGF-β (10 ng/mL) for 30 minutes to analyze SMAD2/3 phosphorylation. Longer treatment (18 hours) was used to test the effects on ECM accumulation in vitro. These studies suggest that TGF-β signaling is dysregulated in human MVP compared with explanted nondiseased mitral valves and to use cultured human VICs to determine the effects of angiotensin II receptor blockers (ARBs) with described TGF-β inhibitory activity on downstream signaling and ECM production.

Isolation and Culture of VICs

VICs were isolated by digesting mitral valve tissue in 125 U/mL collagenase XI, 60 U/mL hyaluronidase I, 60 U/mL Dase 1, and 450 U/mL collagenase I (Sigma-Aldrich) in phosphate-buffered saline at 37°C for 3 hours. They were passaged through a 70-μm cell strainer and then suspended and cultured in M199 medium supplemented with 10% fetal bovine serum, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). After 3 passages, there was no appreciable difference noted in the characteristics or responses of cells derived from diseased or normal valve tissue and both types were used for signaling studies. Unless otherwise mentioned, VICs were treated with TGF-β (1 ng/mL) for 30 minutes for signaling studies and 18 hours for expression analysis.

Histology

Mitral valve tissues were fixed in formalin, embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin, elastin-Van Gieson for elastin, and Sirius red for collagen using standard techniques.

Immunohistochemistry and Immunofluorescence

Immunolabeling of paraffin-embedded sections was performed as previously described. Quantification of specific staining was performed by counting positive cells within 10 random high-power fields (HPFs) divided by the total number of cells using at least 3 independent clinical specimens. All layers of the mitral valve (fibrosa/spongiosa/ventricularis) were assessed and included in quantification of positive cells.

Western Blotting

Proteins were extracted from cultured VICs or frozen valve tissues and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as previously described. Beta-actin was used as a loading control.

Flow Cytometry

Cells were isolated by digestion mitral valve tissue as described previously and analyzed immediately. Cells were labeled with primary and secondary antibodies or isotype-matched, irrelevant IgG and analyzed using a FACSort (BD Biosciences).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

RNA was extracted from cultured VICs or frozen valve tissues as previously described. Samples were analyzed in duplicate and target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase.

Antibodies

The following antibodies were used: COL1A1, COL3A1 (Novus Biologicals); elastin, smooth muscle actin, CD45 (Sigma); TGF-β1/2/3, LAP-β1, VIM, SM22, CD31 (R&D); SMAD2/3, phosphorylated (P)-SMAD2/3, ERK (Santa Cruz); CD163 (Abnova); CD20, CD3 (Abcam); P-ERK1/2, and β-actin (Cell Signaling).

Chemical Inhibitors and Pharmaceuticals

The following chemical inhibitors were used: SB505124 (5 μmol/L; Sigma) for TGFBR1; SIS-3 (3 and 10 μmol/L) for SMAD3; SB203580 (10 μmol/L) for p38; and U0126 (10 μmol/L) and PD98059 (50 μmol/L) for ERK (Calbiochem). VICs were pretreated with chemical inhibitor for 30 minutes before TGF-β. Pharmaceuticals included: losartan (10 μmol/L), candesartan (10 μmol/L, Cayman Chemicals), and telmisartan (10 μmol/L, Sigma). Cultured cells were grown in 6-well plates and treated with inhibitors or pharmaceuticals for 30 minutes before addition of TGF-β.

Statistical Analysis

Unpaired Student t test was used to compare means of 2 experimental groups. One-way analysis of variance with Newman-Keuls or 2-way analysis of variance with Bonferroni post hoc analysis was used to compare >2 experimental groups. In cases in which N=3 and concerns were of heterogeneous variance, the data were transformed using natural logarithm (Figures 1F, 2C, 3B, 3C, and 4A). Probability values were 2-tailed and probability values <0.05 were considered to indicate statistical significance. Data presented are mean±SEM. Statistical analyses were performed using Prism 4 (GraphPad Software).

Results

ECM Accumulation Correlates With Increased TGF-β Expression and Signaling in Myxomatous Mitral Valves

Histological examination demonstrated thickening of diseased valve leaflets, abundance of collagen fibers, and dis-
ruption of elastin in diseased mitral valves when compared with normal valve tissue (Figure 1A). Increased accumulation of ECM components collagen (COL1A1 and COL3A1) and elastin in diseased tissue was confirmed by quantitative polymerase chain reaction (Figure 1B). The expression of all 3 TGF-β subtype transcripts was increased significantly in diseased tissue, which was confirmed by immunoblotting (Figure 1C–D). There was increased labeling for mature TGF-β in diseased tissue by immunohistochemistry, but the staining was inconsistent between specimens. However, latency-associated peptide (LAP), the precursor remnant that is noncovalently bound to mature TGF-β and obscures its
Serological detection, was consistently increased in diseased tissue (74.1% ± 1.8% of cells per 10 HPF, N = 3) as compared with normal valve tissue (25.8% ± 1.9% of cells, P < 0.001) by immunohistochemistry and was selected for forthcoming analyses (Figure 1E). Fluorescence-activated cell sorter analysis confirmed a marked difference in surface LAP expression (Figure 1F).

Expression of TGF-β receptor 1 was upregulated in diseased tissue by quantitative polymerase chain reaction (Figure 2A) and canonical TGF-β signaling was upregulated in diseased tissue demonstrated by significantly more abundant immunostaining for phosphorylated SMAD2/3 in diseased tissue (65.3% ± 1.8% of cells per 10 HPF, N = 3) as compared with normal valve tissue (30.3% ± 2.7% of cells, P < 0.05), whereas no difference was noted in total SMAD2/3 immunostaining (Figure 2B–C).

Valvular Interstitial Cells Are the Primary Producers and Responders of TGF-β in Myxomatous Mitral Valves

VICs are characterized by vimentin (VIM) staining, which was homogeneously expressed and VIM+ cells represented the majority of cells within mitral valves with no appreciable difference between normal and diseased tissue (Figure 2D). In contrast, expression of smooth muscle actin was markedly increased in diseased valve tissue (68.5% ± 1.3% of cells per 10 HPF, N = 3) as compared with normal valve tissue (11.0% ± 0.9% of cells, P < 0.001). Infiltration of CD45+ leukocytes was increased in diseased (6.8% ± 0.5% of cells per 10 HPF, N = 3) compared with normal valve tissue (3.2% ± 0.3%, P < 0.001). CD45+ cells were not uniformly distributed throughout the valve tissue, but rather were present in clusters and comprised primarily of CD163+ macrophages and CD3+ T cells with few CD20+ B cells (online-only Data Supplement Figure I).

Almost all cell types secrete TGF-β to a varying degree and expression of LAP on the cell surface can indicate the source of TGF-β.17 Immunofluorescence demonstrated co-staining for LAP in the majority of VIM+ cells in diseased tissue (Figure 3A). This was confirmed by fluorescence-activated cell sorter, in which a significantly higher number of VIM+ LAP+ cells were noted in diseased compared with normal tissue (Figure 3B). Relatively few CD45+ cells expressed surface LAP (Figure 3C). Immunofluorescence

Figure 2. Increased TGF-β signaling and evidence of VIC activation in myxomatous mitral valves. Relative expression determined by qPCR of (A) TGF-β receptor Types 1 and 2 (TGFBR1 and 2), N = 10 for normal; N = 6 for diseased, t test. Immunohistochemistry (B) and immunolabeling frequency (C) for SMAD2/3 and P-SMAD2/3, average of 10 random HPF, N = 3, t test. Increased number of activated VIC and inflammatory cells in diseased tissue demonstrated by (D) immunohistochemistry for VIM, SMA, and CD45 (scale bar 100 μm). Immunolabeling frequency, N = 3. *P < 0.05, ***P < 0.001. TGF indicates transforming growth factor; VIC, valvular interstitial cell; qPCR, quantitative polymerase chain reaction; HPF, high-power field; SMA, smooth muscle actin.
Figure 3. Localization of TGF-β ligand and signaling to VIC in myxomatous mitral valves. A, Immunofluorescence of diseased tissue demonstrating colocalization of LAP and VIM. Costaining with: VIM, LAP, and DAPI (upper panel) or CD45, LAP, and DAPI (lower panel). Lower right corner inset shows staining with an isotype-matched irrelevant antibody (scale bar 100 μm). Bar graphs show percentage of double positive cells, average of 10 random HPF, N=3. Representative FACS dot plots show staining for (B) VIM and LAP and (C) CD45 and LAP in cells isolated from normal and diseased mitral valve tissue. Control is nonspecific IgG. Bar graphs show percentage of VIM+LAP+ cells or CD45+LAP+ cells, N=3, t test. D, Immunofluorescence of diseased tissue demonstrating colocalization of P-SMAD2/3 and VIM. Costaining with VIM, P-SMAD2/3, and DAPI (upper panel) or CD45, P-SMAD2/3, and DAPI (lower panel). Bar graphs show percentage of double positive cells, average of 10 random HPF, N=3. ***P<0.001. TGF indicates transforming growth factor; VIC, valvular interstitial cell; LAP, latency-associated peptide; VIM, vimentin; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; HPF, high-power field; FACS, fluorescence-activated cell sorter.
was also performed to identify which cell types displayed activation of canonical TGF-β signaling. The majority of VIM+/SMAD2/3 cells in diseased tissue costained for phosphorylated SMAD2/3, whereas only a small fraction of CD45+/SMAD2/3 cells displayed detectable phosphorylated SMAD2/3 (Figure 3D).

TGF-β Is Sufficient and Necessary for ECM Production in Cultured Mitral Valves
To extend our descriptive observations from clinical specimens, we used a tissue culture system to test the effects of TGF-β in human mitral valves. TGF-β treatment of normal mitral valve tissue induced phosphorylation of SMAD2/3 (Figure 4A). There was significantly increased staining for P-SMAD2/3 in TGF-β-treated tissue (69.6%±2.0% of cells per 10 HPF, N=3) as compared with untreated valve tissue (33.7%±1.5% of cells, P<0.001). TGF-β-induced ECM transcript expression in both normal and diseased tissue (Figure 4B–C). Conversely, serological neutralization of TGF-β resulted in significant downregulation of intrinsic ECM production in diseased tissue without significant effects on low basal ECM expression in normal tissue (Figure 4B–C).

TGF-β Regulates ECM Production by SMAD and p38-Dependent, ERK-Independent Signaling in Cultured VICs
VICs were isolated and propagated from mitral valve tissue. The cells were adherent and formed a monolayer of spindle-shaped slow-growing cells. Immunofluorescence and fluorescence-activated cell sorter analysis confirmed a near-uniform presence of VIM, partial expression of smooth muscle actin, and absence of the leukocyte common marker CD45, endothelial cell marker CD31, and smooth muscle cell marker SM22 (Figure 5A–B). Treatment of VIC with TGF-β resulted in a time- and dose-dependent increase in the relative expression of COL1A1, COL3A1, and elastin transcripts (Figure 5C). Inhibition of TGF-β receptor 1 kinase, SMAD3, and p38 effectively abrogated TGF-β-induced ECM expression, whereas ERK inhibitor did not have any effects (Figure 6A). Additional inhibitors for JNK1 (SP600125), RhoA (Y27632, Lantruculin B), and PP2A (Okadaic acid) were tested but did not have any effects (results not shown). Immunoblotting confirmed that TGF-β induced phosphorylation of SMAD2, SMAD3, and p38 in VIC in a time- and dose-dependent manner. Baseline ERK phosphorylation was not appreciably affected by TGF-β treatment at the times and doses tested (Figure 6B). ERK inhibitors effectively inhibited baseline ERK1/2 phosphorylation, whereas they did not have any significant effects on TGF-β-induced ECM expression (online-only Data Supplement Figure II).

Inhibition of TGF-β Signaling Pathway by ARBs
Treatment of VIC with losartan, candesartan, and telmisartan for 30 minutes before TGF-β stimulation resulted in effective inhibition of TGF-β-induced ECM expression (Figure 6C). Immunoblotting demonstrated that TGF-β-mediated phos-
phorylation of SMAD2, SMAD3, and p38 was prevented by losartan, whereas ERK phosphorylation was not affected (Figure 6D).

**Discussion**

Despite being a relatively common disorder, mechanistic insight into the pathophysiology of sporadic MVP in humans is limited. TGF-β signaling controls diverse cellular processes and plays an important role in development, tissue homeostasis, and various pathological conditions such as cancer, fibrosis, autoimmune, and vascular diseases.18 We were interested in investigating the role of TGF-β in sporadic myxomatous mitral valve disease in humans based on evidence for TGF-β dysregulation in both familial and syndromic MVP as well as in animal and cell culture models.10,12–14 For that purpose, we collected diseased mitral valve tissue from patients undergoing operative repair for severe mitral regurgitation secondary to myxomatous degeneration and made comparisons to normal tissue.

All 3 subtypes of TGF-β, LAP, TGF-β receptor Type 1, and the downstream SMAD pathway were markedly increased in diseased tissue. The ECM components COL1A1, COL3A1, and elastin, well-established TGF-β-responsive genes,19 were also present in significant excess in diseased valves and their production was dependent on TGF-β in cultured tissue supporting the hypothesis that TGF-β is integrally involved in the pathogenesis of MVP. The results demonstrate that TGF-β signaling and profibrotic effects occur in mitral valve tissue and that the excessive expression of ECM molecules in diseased tissue can be effectively inhibited in vitro by TGF-β neutralization.

VICs are the most prevalent cells in mitral valve tissues and are postulated to maintain normal valve function and regulate valve repair and remodeling.20 VICs were the predominant cells expressing and responding to TGF-β in clinical specimens of myxomatous mitral valve disease. They appear to be the primary source of TGF-β ligand as demonstrated by LAP surface expression.17 They are also activated as evidenced by P-SMAD2/3 and VIM costaining as well as expression of smooth muscle actin. This agrees with the concept of potent TGF-β autocrine regulation of VIC in vitro,13,14 but our findings are the first time this is clearly demonstrated in clinical tissues.

Pharmacological experiments demonstrated that TGF-β regulates ECM synthesis in VIC primarily through its canonical SMAD signaling pathway at the same time as also requiring p38 activation but was independent of ERK. This diverges from a recent study on FBN1 mutant mice in which SMAD, ERK1/2, and JNK1 pathways are all involved in aneurysm formation,21 but this may be related to organ-
Figure 6. Involvement of the SMAD2/3 and p38 signaling pathway in TGF-β-induced responses and its inhibition by angiotensin receptor blockers.

A, qPCR demonstrates the effects of chemical inhibitors on TGF-β-mediated ECM expression. Cultured VIC were pretreated with SB505124 (TGFBR1K), SIS-3 (SMAD3), SB203580 (p38), U0126 (ERK1), PD98059 (ERK1), or vehicle (control) before TGF-β treatment. N=3, one-way analysis of variance (ANOVA) with Newman-Keuls post hoc analysis compared with TGF-β.

B, Dose- and time-response to TGF-β demonstrated by Western blots for phosphorylated SMAD2, SMAD3, p38, and ERK1/2 in cultured VIC. Dose response was performed at 1 hour and time response at 1 ng/mL.

C, Relative mRNA levels of ECM in VIC treated with vehicle (control) or TGF-β after pretreatment with losartan, candesartan, or telmisartan, N=3, 2-way ANOVA with Bonferroni post hoc analysis compared with control.

D, Western blotting for SMAD2, SMAD3, p38, and ERK1/2 phosphorylation. VICs were pretreated with or without losartan prior to TGF-β. *P<0.05, **P<0.01, ***P<0.001. TGF indicates transforming growth factor; qPCR, quantitative polymerase chain reaction; ECM, extracellular matrix; VIC, valvular interstitial cell.
species-specific differences or due to different durations of TGF-β signaling activation.

Angiotensin II and TGF-β crosstalk is well described in fibrosis and ECM deposition involving both canonical and noncanonical signaling pathways that can be inhibited by ARB.22 Losartan competitively antagonizes binding of angiotensin II to its Type 1 receptor but also effectively inhibits progression of aortic root aneurysms in both FBN1 mutant mice and patients with Marfan disease through anti-TGF-β effects.23–25 The precise action of ARB on TGF-β signaling is not clearly defined, but recent study in FBN1−/− mice suggests that losartan effectively shunts angiotensin II signaling toward the Type 2 receptor involving both SMAD and ERK.26

We show that in vitro treatment of cultured VIC with ARB effectively inhibits the SMAD and p38 pathways and abrogates TGF-β-induced ECM expression independent of ERK.

In this study, we demonstrate that TGF-β, SMAD2/3 activation, and VIC are crucial mediators in the profibrotic manifestations of MVP that are attenuated by losartan in vitro. Further studies in vivo are warranted to test whether ARB treatment translates into reduction of clinical complications related to MVP. Modulation of the progression of MVP with an established agent of favorable therapeutic profile based on mechanistic considerations would be of great clinical significance.

Sources of Funding

This work was supported by the Department of Surgery, Yale University, Charles W. Oshe Funds Research Grant.

Disclosures

None.

References

Modulation of Transforming Growth Factor-β Signaling and Extracellular Matrix Production in Myxomatous Mitral Valves by Angiotensin II Receptor Blockers
Arnar Geirsson, Mansher Singh, Rahmat Ali, Hussain Abbas, Wei Li, Juan A. Sanchez, Sabet Hashim and George Tellides

*Circulation*. 2012;126:S189-S197
doi: 10.1161/CIRCULATIONAHA.111.082610
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 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/126/11_suppl_1/S189

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/