Periostin as a Novel Factor Responsible for Ventricular Dilation

Naruto Katsuragi; Ryuichi Morishita, MD, PhD; Noriko Nakamura, MD; Tayehito Ochiai, PhD; Yoshiaki Taniyama, MD, PhD; Yasuhiro Hasegawa; Kayoko Kawashima; Yasufumi Kaneda, MD, PhD; Toshio Ogihara, MD, PhD; Keijiro Sugimura, PhD

Background—Periostin is highly expressed in the myocardium in patients with heart failure. However, no report has documented the function of periostin. To identify the function of periostin in the pathophysiology of heart failure, overexpression or loss of function of the periostin gene was examined by direct transfection into the rat heart.

Methods and Results—Rats transfected with the periostin gene by the HVJ-liposome method showed left ventricular (LV) dilation as assessed by echocardiography, accompanied by an increase in periostin expression. Consistently significant differences were observed in LV pressure, LV end-diastolic pressure, LV \( \frac{dP}{dt}_{\text{max}} \), and LV \( \frac{dP}{dt}_{\text{min}} \) at 6 and 12 weeks after transfection in rats transfected with the periostin gene, accompanied by a decrease in cardiac myocytes and an increase in collagen deposition. Importantly, periostin has the ability to inhibit the spreading of myocytes and the adhesion of cardiac fibroblasts with or without fibronectin. Markers of cardiac dysfunction such as brain natriuretic peptide and endothelin-1 gene expression were significantly increased after transfection in the LV of rats transfected with the periostin gene. These data demonstrate that overexpression of the periostin gene led to cardiac dysfunction. Thus, we examined the inhibition of periostin in Dahl salt-sensitive rats by an antisense strategy because periostin is highly expressed in heart failure. Importantly, inhibition of periostin gene expression resulted in a significant increase in survival rate, accompanied by an improvement of LV function.

Conclusion—The present study demonstrates the contribution of the periostin gene to cardiac dilation in animal models. Inhibition of periostin might become a new therapeutic target for the treatment of heart failure. (Circulation. 2004;110:1806-1813.)

Key Words: gene therapy ■ heart failure ■ remodeling ■ adhesion

Currently, chronic heart failure is treated mainly with \( \beta \)-blockers, angiotensin-converting enzyme inhibitors, and angiotensin II type 1a receptor blockers by reducing the excessive load to the heart caused by activation of the sympathetic nervous system and renin-angiotensin system. However, only 20% to 30% of patients with chronic heart failure respond to these agents. Because lifetime recuperation of chronic heart failure patients is still defective, an effective therapy is definitely needed. Recent progress in molecular and cellular biology has identified the mechanism of heart failure. Patients with hypertrophic cardiomyopathy are reported to have mutations in the cardiac \( \beta \)-myosin heavy-chain (MHC), cardiac troponin T, or cardiac myosin-binding protein C genes. One cause of dilated cardiomyopathy has been identified as a mutation of the dystrophin gene, which encodes a cell membrane–lining protein, or the tafazzin gene, which encodes a cell membrane–lining protein, or the tafazzin gene. In animal models, heart failure has been induced by knockout of the dystrophin, \( \alpha \)-MHC, muscle LIM protein, or angiotensin II type 1a receptor genes or by overexpression of the leukocyte tyrosine kinase, calcinurin, nuclear factor of activated T cell 3, or Gs genes, to name a few. Thus, knowledge of the mechanisms underlying heart failure is still limited.

Recently, to identify unknown mechanisms involved in the process of heart failure, gene expression analysis with new tools such as subtractive hybridization, differential display, or microarray techniques has been a major point of interest. As a result, the periostin gene was identified to be highly expressed in a heart failure model. Periostin was previously screened from a mouse osteoblastic cell line MC3T3-E1 cDNA library and is known to be expressed in bone and to a lesser extent in lung but not in other tissues. The periostin gene encodes a protein comprising 838 amino acids and has a typical signal sequence, followed by a cysteine-rich domain, a 4-fold–repeated domain, and a C-terminal domain. Until now, the physiological functions of periostin have not been understood, except for one report that suggests that periostin might modulate new bone formation and cell adhe-
sion. To identify the role of periostin in heart failure, we used the local overexpression and loss-of-function approaches in the present study.

Methods

Transfection of Periostin Gene Into Rat Heart

The rat homologue of mouse periostin cDNA was cloned from a commercially available source (Clontech). Rat periostin cDNA with the coding sequence of 838 amino acids was inserted into a plasmid containing the cytomegalovirus promoter to generate pcDNA1/tpP. The vector used as a control was the cytomegalovirus expression vector plasmid that did not contain rat periostin cDNA.

We used a high-efficiency transfection method with hemagglutinating virus of Japan (HVJ)–coated liposomes to transfect the myocardium. Male Sprague-Dawley rats (360 to 380 g; Charles River Laboratories, Atsugi City, Japan) were anesthetized by injection with sodium pentobarbital (50 mg/kg IP). Rats were intubated and connected a respirator. The HVJ-liposome complex, injection with sodium pentobarbital (50 mg/kg IP). Rats were anesthetized with sodium pentobarbital (30 mg/kg IP). The left lateral thoracotomy. The injection volume of HVJ-liposome directly into the apex of the heart with a 30-gauge needle through a

Echocardiographic and Hemodynamic Studies

Rats were lightly anesthetized with ketamine HCl (50 mg/kg IP) and xylazine (10 mg/kg IP) at 3, 6, and 12 weeks after transfection. The echocardiographic system was equipped with a 7.0-MHz transducer (Core Vision Pro SSA-350 A, Toshiba). Two-dimensional, short-axis views of the left ventricle (LV) were obtained. M-mode tracing was recorded through the anterior and posterior LV walls. Anterior and posterior end-diastolic and end-systolic wall thicknesses and LV diastolic and systolic internal dimensions were measured.

Three days after the final echocardiogram, rats were lightly anesthetized with sodium pentobarbital (30 mg/kg IP). The left carotid artery was then cannulated with a fluid-filled, PE50 catheter. Heart rate was obtained from the arterial pressure pulse. After arterial blood pressures were recorded, the cannula in the left carotid artery was briefly advanced into the LV under constant pressure monitoring to evaluate LV systolic and end-diastolic pressures. After completion of hemodynamic measurements, the heart was rapidly removed. The LV was separated from the atria and right ventricle with an incision along the septum from the base to the apex. After being weighed, the tissues were rapidly frozen in LN₂ and stored at −80°C until use.

Real-Time, Quantitative RT-PCR

Total RNA was extracted from the LV and septum at 3, 6, or 12 weeks after transfection. At 2 and 6 days after transfection, total RNA was also extracted for periostin gene determination with ISOGEN (Nippon Gene). Transcripts of mRNA were quantified by the dual-labeled fluorogenic probe method for real-time reverse transcription–polymerase chain reaction (RT-PCR) with use of a Prism 7700 thermal cycler and sequence detector (Perkin-Elmer/ABI).

RT-PCR Primers and Probes

The RT-PCR primers and probes were as follows: (1) Rat periostin sense, 5'-TGCAAAGAAGGCAGCTCCAAAGGT-3'; 5'-TATCTATACGACGTGTTGAGG-3' (nucleotides 652 to 674); antisense, 5'-GGATCCGTTTTACCGAGATG-3'; 5'-AGGAGCCATACGAAAGCAGCATAGACGCT-3' (nucleotides 590 to 592); (2) rPN-CAGGGGCTGAAGATGGTTCCT-3' (nucleotides 216 to 237); antisense, 5'-AAGGCG CCTGAATTTGCAACGC-3'; 5'-AGGAAGCAGCTCCGACCCCT-3' (nucleotides 5777 to 5794); (3) antisense, 5'-CCFAAGTTGTCTTGTTCAAGCCG-3' (nucleotides 5869 to 5890); 5'-FAM-TAGATCTGGTCTGAC CTCCACCTAAGGATG-3' (nucleotides 5816 to 5845); and (7) glyceraldehyde 3-phosphate dehydrogenase (GenBank accession No. AF106860) as a constitutive control; sense, 5'-TGCCACCCACTTCTCGCTTG-3' (antisense 471 to 489); antisense, 5'-GGATCCGAGGATGATGTTCG-3'; 5'-FAM-CAGAAAGCAGCTCCGATGCAGCCCTC-3' (antisense ODN was composed of several oligonucleotides), fibronectin (10 μg/mL, Sigma), nonpurified baculovirus expression system (In-vitrogen). In brief, a hexahistidine-tagged fusion construct was cloned into the pFastBac1 plasmid. The isolated recombinant plasmid was transformed into Escherichia coli DH10BAC cells harboring a baculovirus shuttle vector, and white colonies representing the clones that had undergone transposition were isolated. High-molecular-weight DNA was prepared from the isolated clones and used to transfect SF-9 cells. Recombinant baculovirus was prepared as a conditioned culture medium and was used to infect 10% cells at a multiplicity of infection of 1. Periostin was produced in serum-free medium from SF-9 cells.

Solid-Phase Binding Assay of Cell Adhesion of Myocytes and Cardiac Fibroblasts

Primary cultures of neonatal myocytes and fibroblasts were prepared from 2-day-old Wistar-strain rats. Cells were added to the 24-well plates that had been coated with nonpurified recombinant periostin mRNA on the basis of the intensity of inhibition in COS-1 cells transfected with rat periostin plasmid expression.

Male Dahl salt-sensitive rats (Dahl S/Jr Sea; Seac Yoshitomi, Ltd, Chikujyo-gun, Fukuoka prefecture, Japan) were fed a high-salt diet (8% NaCl; Nosan Corp) from 6 weeks of age. HVJ-liposome complex containing antisense or sense ODN (15 μmol/L each) was transfected into the hearts of all rats at 11 weeks of age, as described earlier.

Preparation of Recombinant Rat Periostin From SF-9 Cells

A C-terminal hexahistidine-tagged periostin was expressed in insect SF-9 cells with the Bac-to-Bac baculovirus expression system (Invitrogen). In brief, a hexahistidine-tagged fusion construct was cloned into the pFastBac1 plasmid. The isolated recombinant plasmid was transformed into Escherichia coli DH10BAC cells harboring a baculovirus shuttle vector, and white colonies representing the clones that had undergone transposition were isolated. High-molecular-weight DNA was prepared from the isolated clones and used to transfect SF-9 cells. Recombinant baculovirus was prepared as a conditioned culture medium and was used to infect 10% cells at a multiplicity of infection of 1. Periostin was produced in serum-free medium from SF-9 cells.
per well were counted. To demonstrate the specificity of inhibition of cell binding by periostin, the coated wells were precoated with fibronectin (10 μg/mL) at 37°C for 1 hour before the adhesion assay was performed.

**LDH and MTS Assay**

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released on cell lysis. LDH released to the culture medium by dead cells as well as LDH contained in living cells was measured with the CytoTox96 kit (Promega). This assay is based on the LDH-catalyzed oxidation of lactate to pyruvate in the presence of NAD, which is reduced to NADH. The formation of NADH is coupled to the reduction of a tetrazolium salt, measured photometrically at 490 nm on a 96-well plate reader (Molecular Devices).

MTS is a tetrazolium salt that is bioreduced by cells into a colored formazan product that is directly proportional to the number of living cells in culture. The MTS assay assesses cell viability by measuring mitochondrial function and was performed with the CellTiter96 aqueous one-solution cell proliferation assay kit (Promega). The quantity of formazan product was measured by the amount of absorbance at 490 nm.

**Statistical Analysis**

All results are expressed as mean±SEM. Data were analyzed by Student t test after ANOVA, except for analysis of survival rate and hemodynamics. P<0.05 was considered statistically significant.

**Results**

**Transfection of Periostin Gene Into Rat Hearts**

To confirm successful transfection of the periostin gene, the myc sequence tagged to the rat periostin gene expression plasmid, which allows endogenous rat periostin mRNA to be distinguished, was used for transfection into the rat heart. Endogenous periostin was highly expressed in fibroblasts, whereas little expression could be detected in myocytes (Figure 1A, P<0.01). Expression of exogenously expressed periostin, as well as endogenous periostin, was mainly observed in the septum and LV (Figure 1B). The expression level of the transfected gene was analyzed by real-time, quantitative RT-PCR with specific primers encoding the rat periostin and myc regions. Expression of the exogenous periostin gene, with the c-myc tag in the myocardium transfected with periostin gene, could be detected from 2 days to 12 weeks after transfection (P<0.01, Figure 1C). Its expression level was extremely high at 2 days after transfection and decreased after that time. Interestingly, the total amount of periostin gene expression was also increased from 2 days to 6 weeks after transfection in myocardium transfected with the periostin gene compared with control vector (P<0.01, Figure 1D). It is probable that exogenously added periostin might enhance expression of the endogenous periostin gene expression in rats transfected with periostin gene (2 days, n=6; 6 days, n=6; 3 weeks, n=4; 6 weeks, n=4; 12 weeks, n=12); control, amount of periostin gene expression in rats transfected with control gene (2 days, n=4; 6 days, n=4; 3 weeks, n=4; 6 weeks, n=4; 12 weeks, n=10). *P<0.01 vs control. Other abbreviations are as defined in text.
ostin gene. Although there was no significant difference in body weight, heart weight, LV weight, heart rate, and mean blood pressure between rats transfected with control vector and the periostin gene up to 12 weeks after transfection, cardiac function was significantly altered (Figure 2). LV systolic pressure was significantly decreased in rats transfected with the periostin gene from 3 to 12 weeks after transfection (\( P < 0.05 \)). Similarly, LV \( dP/dt_{\text{max}} \) as well as LV \( dP/dt_{\text{min}} \) was significantly decreased to 72% and 76%, respectively, in rats transfected with the periostin gene at 6 weeks after transfection and continued up to 12 weeks after transfection. In contrast, LV end-diastolic pressure as a diastolic parameter in rats transfected with the periostin gene was significantly increased to 640% at 6 weeks after transfection (\( P < 0.01 \)). These data clearly demonstrate that overexpression of the periostin gene led to cardiac dysfunction.

**Echocardiographic Evaluation of LV Function**

To confirm cardiac dysfunction induced by periostin gene transfer, we also analyzed cardiac structure by means of echocardiography. As shown in Figure 3A, M-mode echocardiograms demonstrated dilation of the ventricles transfected with the periostin gene compared with control vector. Anterior and posterior wall thicknesses in diastole of the rat hearts transfected with the periostin gene were not different from those in control rats at 3 weeks after transfection, but both were significantly less in rats transfected with the periostin gene than in those transfected with control vector at 12 weeks after transfection (\( P < 0.01 \)). Both LV diastolic cavity size and LV systolic cavity size were significantly increased in rats transfected with the periostin gene from 3 to 12 weeks after transfection (\( P < 0.01 \)). Consistently, fractional shortening was significantly decreased at 12 weeks.
weeks after transfection in rats transfected with the periostin gene ($P<0.01$).

Cardiac dysfunction induced by periostin was also confirmed by histological studies. As shown in Figure 4A, microscopic analysis demonstrated a significant decrease in cross-sectional diameter of cardiac myocytes of rats transfected with the periostin gene at 3 weeks after transfection compared with control vector ($P<0.05$). A significant decrease in the size of myocytes continued for up to 12 weeks after a single transfection. In addition, accumulation of interstitial collagen was markedly enhanced in myocardium transfected with the periostin gene compared with control vector from 3 to 12 weeks after transfection (Figure 4B). The features of heart failure were also confirmed by upregulation of cardiac gene expression in response to cardiac dysfunction. BNP mRNA expression was significantly increased by 50% to 80% in the hearts of rats transfected with the periostin gene from 3 to 12 weeks after transfection ($P<0.01$, Figure 4C). Expression of the ET-1 gene in the hearts of rats transfected with the periostin gene was also increased 2.2- to 5.4-fold compared with control rats from 3 to 12 weeks after transfection. $\alpha$-MHC mRNA expression in rats transfected with the periostin gene was decreased 80% compared with control rats at 6 weeks after transfection, whereas $\beta$-MHC mRNA expression in rats transfected with the periostin gene was decreased 60% at 3 weeks after transfection.

Moreover, to address the molecular mechanisms whereby periostin impaired cardiac function, we used an in vitro culture system. We particularly focused on the effects of periostin on cell spreading or attachment. First, we examined the cell spreading of myocytes on plates coated with fibronectin, periostin, and BSA. The coating with periostin inhibited the spreading of myocytes, whereas fibronectin increased the spreading compared with BSA (Figure 5A, $P<0.01$). Interestingly, addition of periostin significantly inhibited the spreading of myocytes induced by the coating with fibronectin (Figure 5B, $P<0.05$). Next, we examined the attachment of cardiac fibroblasts on the plates coated with fibronectin, periostin, and BSA. Interestingly, as shown in Figure 5C, cardiac fibroblasts did not attach to the plates coated with periostin but did to those coated with fibronectin or BSA ($P<0.01$). We also examined the effects of periostin on the adhesive activity of fibronectin. Attachment of cardiac fibroblasts to the plates coated with fibronectin was significantly inhibited by periostin but not by fibronectin or BSA (Figure 5D, $P<0.01$). In contrast, cPLA2 as a negative control did not affect cell attachment. These results demonstrate that periostin has the ability to inhibit adhesion of cardiac fibroblasts, with or without fibronectin.

Death of myocytes as well as of cardiac fibroblasts was assessed by the LDH assay. However, periostin did not affect the number of
myocytes and fibroblasts. In addition, apoptosis could not be detected in both cells by the terminal dUTP nick end-labeling assay. Finally, we examined the effects of periostin on cell growth. As shown in Figure 5E, periostin did not inhibit cell proliferation of fibroblasts, but cell attachment was inhibited by periostin.

**Improvement in Survival Rate and Hemodynamics by Inhibition of Periostin**

Finally, we used an antisense strategy to prove the contribution of periostin to cardiac failure. Thus, we examined whether the survival rate of Dahl salt-sensitive rats could be improved by inhibition of endogenous periostin by using an antisense ODN against rat periostin. We chose Dahl salt-sensitive rats as a model of heart failure because periostin mRNA is highly expressed at the stage of heart failure in these rats (Figure 6A). The specificity of antisense ODN was confirmed by in vitro experiments (Figure 6B), because only antisense ODN, but not sense ODN, inhibited periostin mRNA expression at a concentration of 40 nmol/L. Unexpectedly, the survival rate of rats transfootected with antisense ODN was significantly higher than that in sham-operated rats and in rats transfected with sense ODN (P<0.01, Figure 6C). At 43 days after transfection, LV dP/dt max and LV dP/dt min were significantly improved in rats transfected with antisense ODN compared with the sham and sense ODN groups (P<0.01, Figure 6D and 6E).

**Discussion**

LV dilation is a predictor of survival in humans with heart failure, such as that due to ischemic heart failure, hypertension, valvular heart disease, or dilated cardiomyopathy. Therefore, the progression of LV dilation leads to severe heart failure. Although numerous factors have been reported to be involved in LV hypertrophy, only a few have been identified to contribute to dilation. It is important that this study clearly demonstrated the contribution of periostin to the development of chronic heart failure through cardiac dilation. A single transfection of the periostin gene resulted in a significant increase in LV diastolic and systolic internal dimensions at 3 weeks after transfection. In addition, fractional shortening was also significantly decreased at 12 weeks after transfection. Another interesting aspect of the present study was the decrease in wall thickness. Hemodynamic study demonstrated a significant difference in LV pressure at 3 weeks after transfection and in LV end-diastolic pressure, LV dP/dt max, and LV dP/dt min at 6 weeks after transfection. These results reveal an aggravation of contractile function by the transfection of periostin. It is quite surprising that a single transfection of a single gene altered cardiac function, although we used the HVJ-liposome method as a highly efficient transfection method into the myocardium for in vivo gene transfer. Nevertheless, the expression level of periostin in rats transfected with the exogenous periostin gene continued to be increased for up to 6 weeks, even with only one transfection. Importantly, the total amounts of exogenous and endogenous periostin seemed to be much higher than a simple additive effect. It is probable that the expression level of endogenous periostin was upregulated by the transgene in an autocrine-paracrine manner. Alternatively, other factor(s) might control the expression level of endogenous periostin.

Importantly, the inhibition of periostin by antisense ODN resulted in an improvement of cardiac function in an animal model of cardiac heart failure in which cardiac periostin expression was increased at the stage of heart failure. Therefore, periostin is an important factor for the development of heart failure. Because transgene expression was transient in

---

Figure 5. Inhibition of spreading of myocytes or attachment of cardiac fibroblasts to plates by periostin. A, Inhibition of cell spreading of myocytes on plates by periostin. Primary rat myocytes were added to 24-well plates (150 000/well) coated with recombinant periostin (PN), fibronectin (FN), or BSA and then incubated at 37 °C for 72 hours. Then, area of cells was measured with NIH Image software. *P<0.05, **P<0.01 vs fibronectin. B, Inhibition of cell spreading of myocytes on plates with fibronectin by periostin. Plates were precoated with fibronectin. Then, plates were again coated with recombinant periostin (PN), fibronectin (FN), or BSA at 10 μg/mL. **P<0.05 vs fibronectin. C, Inhibition of cell adhesion of fibroblasts to plates by periostin: Primary rat cardiac fibroblasts were added to 24-well plate (50 000/well) coated with recombinant periostin (PN, ○), fibronectin (FN, △), or BSA (◊) and then incubated at 37 °C for 1 to 3 hours. Then number of attached cells was counted. *P<0.01 vs fibronectin or BSA. D, Inhibition of cell adhesion of fibroblasts to plates with fibronectin by periostin. Plates were again precoated with fibronectin. Then plates were then incubated at 37 °C for 72 hours of incubation on periostin (PN), fibronectin (FN), or BSA (n=8). *P<0.05, **P<0.01 vs fibronectin. Other abbreviations are as defined in text/
this experiment, long-term expression of periostin might enhance cardiac dysfunction more dramatically.

Cardiac dysfunction induced by periostin was followed by changes in markers for cardiac heart failure. BNP is predominantly synthesized in the heart and is secreted from the ventricle as a useful marker of heart failure. The present study demonstrated that BNP gene expression was significantly increased by transfection of the periostin gene from 3 to 12 weeks after transfection. A previous study clearly demonstrated that BNP sharply responded to an aggravation of LV function compared with atrial natriuretic peptide.24 In addition, another useful marker of heart failure, ET-1, was also significantly increased by transfection of the periostin gene. In contrast, changes in specific genes related to the phenotypes of contractile proteins, such as α-MHC and β-MHC mRNA, were minimal. The discrepancy in the features of cardiac dysfunction as assessed by echocardiography and serum heart failure markers and the phenotypic markers of contractile proteins was not clarified in this study.

Unfortunately, our understanding of the mechanisms by which periostin led to heart failure is limited. Because periostin is abundant in the extracellular matrix,20 intimal hyperplasia,22 aneurysm,23 muscle regeneration,25 and cancer,26,27 we speculate that periostin has a role in cell-cell binding and attachment. In addition, periostin was reported to be induced by transforming growth factor-β (TGF-β).28 Moreover, the 4-repeated domain of periostin shows homology with insect fasciclin I,20 a protein implicated in neuronal cell-cell adhesion,29 and with human big-h3,30 a protein induced by TGF-β binding to various collagens.31 On the other hand, periostin has been reported to suppress cancer cell growth.32 Therefore, one might assume that periostin has an inhibitory activity on integrin in myocytes and/or cardiac fibroblasts, and still more binding to cells and collagen may cause the side-to-side slippage of cells, which occurs in association with ventricular dilation after myocardial infarction. This possibility might be supported by the observation that microscopic analysis demonstrated a significant decrease in cross-sectional diameter of cardiac myocytes of rats transfected with the periostin gene compared with control vector. In addition, the present study demonstrated that
periostin inhibited cell adhesion of cardiac fibroblasts to the plates coated or not with fibronectin. Probably the decrease in cell adhesion may also explain the present data. Alternatively, it is also possible that periostin induced by TGF-β may contribute to ventricular remodeling and decompensated eccentric hypertrophy through ET upregulation. Further studies are necessary to identify the molecular mechanism of LV dilation induced by periostin.

Alternatively, inhibition of periostin is important for developing new treatment strategies for patients with heart failure, because apparent prolongation of survival and an improvement in hemodynamics were achieved with antisense ODN. Unfortunately, inhibition of periostin by a single transfection of antisense ODN revealed limited duration of the prolongation of survival. In addition to antisense ODN, development of a neutralizing antibody might become a new potential treatment for heart failure as well as a diagnostic tool.

Acknowledgments
This work was partially supported by a grant-in-aid from the Organization for Pharmaceutical Safety and Research; a grant-in-aid from the Ministry of Public Health and Welfare; a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government.

References

Periostin as a Novel Factor Responsible for Ventricular Dilation
Naruto Katsuragi, Ryuichi Morishita, Noriko Nakamura, Tayehito Ochiai, Yoshiaki Taniyama,
Yasuhiro Hasegawa, Kayoko Kawashima, Yasufumi Kaneda, Toshio Ogihara and Keijiro
Sugimura

Circulation. published online September 20, 2004;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. 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/early/2004/09/20/01.CIR.0000142607.33398.54.citation

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/