Liposome-Mediated Gene Transfection of Endothelial Nitric Oxide Synthase Reduces Endothelial Activation and Leukocyte Infiltration in Transplanted Hearts

Akiko Iwata, PhD; Sadahiro Sai, MD; Yoshio Nitta, MD, PhD; Megan Chen, BA; Riedica de Fries-Hallstrand, PhD; Joy Dalesandro, MD; Robert Thomas, BA; Margaret D. Allen, MD

Background—During cardiac ischemia-reperfusion injury, neutrophilic infiltration of the myocardium is mediated by adhesion molecule expression on activated coronary endothelium. Nitric oxide inhibits neutrophil adhesion to endothelium in vitro by blocking the nuclear factor (NF)-κB–dependent transcription of adhesion molecules. We investigated whether intraoperative gene delivery of endothelial nitric oxide synthase (eNOS) into donor hearts before transplantation would have a similar effect on an entire organ.

Methods and Results—In an allogeneic rabbit heart transplant model, liposomes complexed to the gene encoding eNOS were infused into the donor coronary circulation before transplantation. By 24 hours after transplantation, calcium-dependent nitrite production was significantly higher in eNOS-transfected donor hearts than in the 3 control groups: donor hearts transfected with empty plasmids alone, donor hearts treated with diluent only, and untransplanted native hearts. Intramyocardial neutrophil and T-lymphocyte populations were halved in eNOS-transfected hearts compared with control donor hearts (P<0.05). Moreover, the prevalence of NF-κB activation in microvascular endothelial cells and surrounding cardiac myocytes as well as endothelial vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 expression were all significantly reduced in eNOS-transfected hearts compared with control donor hearts (P<0.01). Without immunosuppression, eNOS-transfected hearts survived longer than controls.

Conclusions—Intraoperative liposome-mediated gene delivery of eNOS to donor hearts can result in early gene expression sufficient to reduce ischemia-reperfusion injury by inhibiting NF-κB activation, adhesion molecule expression, and the early infiltration of leukocytes, all of which may improve graft survival. (Circulation. 2001;103:2753-2759.)

Key Words: gene therapy ■ nitric oxide ■ cell adhesion molecules ■ ischemia ■ reperfusion ■ transplantation

Ischemia-reperfusion injury is the major cause of early mortality after cardiac transplantation and a risk factor in the >300 000 nontransplant cardiac surgical operations performed each year in the United States. Reperfusion after myocardial ischemia results in activation of coronary microvascular endothelial cell adhesion molecule expression, attraction of neutrophils to the myocardium, and subsequent myocardial injury.1 In clinical cardiac transplantation, the ischemic period of 1 to 4 hours between donor heart procurement and implantation affords a unique opportunity to intervene in this chain of events before reperfusion. Similarly, during routine cardiac surgery, the aortic cross-clamp time during cardiopulmonary bypass offers a similar period during which an intraoperative intervention could be performed before reperfusion of the heart.

Nitric oxide (NO) has been shown, both in vitro and in vivo, to inhibit cytokine-induced endothelial expression of adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1], intracellular adhesion molecule-1 [ICAM-1], and E-selectin) and proinflammatory cytokines by the induction, stabilization, and nuclear translocation of IκBα, which, in turn, blocks activation of the proinflammatory transcription factor nuclear factor-κB (NF-κB).2,3 Because leukocyte recruitment to activated endothelium is dependent on adhesion molecule receptor-ligand interactions, gene therapy approaches that increase NO production might inhibit donor endothelial adhesion molecule expression and reduce host leukocyte infiltration into the transplanted organ. The strategic questions to address here are whether our currently inefficient gene delivery systems can produce sufficient gene product to have a therapeutic effect on an entire organ and whether gene expression can be achieved within the short time frame necessary to mitigate very early injury patterns. Because NO can diffuse to surrounding cells, gene delivery of NO synthase (NOS) might be effective even if relatively few cells expressed the gene product. We used the gene
encoding endothelial (eNOS) rather than inducible (iNOS) 
NOS because its calcium dependency and potential for 
feedback control of iNOS production1 might allow some 
homeostatic physiological control as protection against NO 
overproduction. Our laboratory has previously shown that 
intraoperative ex vivo delivery of cationic liposomes to the 
 donor heart before transplantation is quite feasible1 and 
results in histological evidence of reporter gene expression in 
the donor heart by at least 6 hours after transfection. Because 
we and others have also shown that de novo production of the 
integrin adhesion molecules after transplantation requires a 
similar window of time,5,6 gene products may be produced 
within enough time to mitigate even ischemia-reperfusion 
injury.

We recently reported that liposome delivery of reporter 
genes to rabbit carotid arteries targets primarily endothelial 
cells; gene product expression, evident histologically in 3% 
of endothelial cells, was still persistent at 21 days.7 When 
liposome gene delivery of eNOS was applied to transplanted 
carotids, the prevalence of eNOS-expressing cells and NO 
production was doubled, endothelial NF-κB activation and 
adhesion molecule expression was suppressed, and vessel 
wall iNOS expression decreased 10-fold. ENOS gene delivery 
significantly reduced neointima formation in these trans- 
planted arteries.7

To assess the efficacy of intraoperative gene delivery in 
cardiac transplants, in this study we assayed eNOS- 
transfected donor hearts 24 hours after transplantation for 
evidence of NO production, NF-κB activation, endothelial 
ICAM-1 and VCAM-1 expression, and neutrophil and 
T-lymphocyte infiltration. Finally, we asked whether single- 
dose pretransplant gene transfection would extend cardiac 
graft survival without other immunosuppression.

Methods

Animals

New Zealand White rabbits and Stauffland rabbits (R and R 
Rabbitry, Stanwood, Wash) were used for all experiments. All 
experiments were performed in accordance with the Principles 
of Laboratory Animal Care, National Society for Medical Research, 
and the Guide for the Care and Use of Laboratory Animals, Institute 
of Laboratory Animal Resources, National Academy of Science 
(NIH publication 86-23, revised 1985).

Cardiac Transplantation

Our model of heterotopic cervical heart transplantation between 2 
outbred strains of rabbits produces well-characterized early neutro-

phil infiltration as well as allogeneic transplant cellular and vascular 
rejection. Briefly, both donor and recipient animals were anesthe-
tized with a mixture of ketamine (35 mg/kg), xylazine (5.0 mg/kg), 
and atropine (0.032 mg/kg); intubated; and ventilated with halothane in 100% O2. After systemic heparinization (300 U/kg), donor hearts 
were arrested with cold (4°C) nonionic Stanford cardioplegia solu-
tion. Three milliliters of liposome-DNA complexes diluted in 10 mL 
of D2W (final concentration of 0.45 mg DNA/mL) was injected into the 
aortic roots of the donor hearts to perfuse the coronary circulation. 
At implantation, the donor ascending aorta and pulmonary 
artery were anastomosed to the recipient carotid artery and jugular 
vein, respectively. Before reperfusion, the coronary circulation was 
flushed with heparinized D2W to remove excess, nonadherent 
liposomes. The ischemic time of the donor hearts from procurement 
to reperfusion after implantation averaged 32±5 minutes (mean±SEM). Recipient rabbits were allowed to fully recover and 
then were euthanized 24 hours or 7 days later for assessment of both 
the transplanted (donor) and the native (recipient) hearts.

In the graft survival experiments, transplanted hearts were as-
essed daily for heartbeat until asystole. No immunosuppression was 
given.

Preparation of Liposome-DNA Complexes

The transfected plasmids were either a pUC19 control plasmid 
without a functional gene or the plasmid carrying a chloramphenicol 
acetyltransferase (CAT) reporter gene, or a human eNOS—
expressing DNA clone. Liposomes (Valentis Inc) and cationic lipid/DNA 
complexes were prepared as described previously, producing a 
final concentration of 1.5 mg/mL plasmid DNA, 1.5 mmol/L 
cholesterol, and 1.5 mmol/L 1-[2-[9-(Z)-octadecenoyloxy]-2-
[(Z)-heptadecenyl]-3-[hydroxyethyl] imidazolinium chloride 
(BODAI).

With regard to reporter gene pilots, we have previously shown that 
liposome-CAT gene delivery to donor hearts results in CAT gene 
expression in all myocardial distributions at 24 hours after transplan-
tation. Here, 7 additional donor hearts treated with liposome-CAT 
complexes were examined 7 days after transplantation; CAT enzyme 
activities in homogenized specimens from donor and native hearts 
were assayed by liquid scintillation scanning and reported as counts 
per minute per milligram total protein (cpm/mg).4

![Graph](image-url)

**Figure 1.** Nitrite production by aortic and coronary sinus vascular specimens at 24 hours. Mean nitrite production 
(nmol/mg tissue) by Griess reaction at 24 hours in donor hearts transplanted by 
aortic root infusion of liposomes complexed to eNOS gene or pUC control 
plasmids (no functional gene), compared with untransplanted native hearts in the 
same animals. Solid and open bars signify specimens that were incubated in 
the presence or absence of calcium ionophore, respectively, to differentiate 
NO produced by calcium-inducible 
eNOS from calcium-independent iNOS. Bars represent mean values; error bars 
represent SEM. *P<0.01 vs untransplanted native hearts (no functional gene). 
Bars represent mean values; error bars represent SEM. **P<0.01 vs native heart aortic and coronary sinus 
specimens from the same rabbits incubated with calcium ionophore (endorge-
Experimental Groups

Eighteen donor hearts were perfused with liposomes complexed to the eNOS gene: 10 were excised at 24 hours after transplantation, 2 at 7 days for histology, and 6 followed to graft asystole. Three different sets of controls were performed for comparison with 24-hour eNOS-treated hearts. Five donor hearts were treated with the same liposomes complexed to the pUC19 plasmid and promoter sequence without a functional gene; 5 donor hearts were treated with the diluent, D2W, alone; and 5 untransplanted native hearts, without ischemia or reperfusion injury, served as an internal control for the effects of systemically circulating cytokines. Eight other transplanted control hearts, without liposome gene delivery, were followed out to graft asystole (n=6) or excised at 7 days (n=2) for histology.

Nitrite Production

To quantify gene product expression by vascular cells alone, without surrounding myocardium, nitrite production was measured by the Griess reaction in 5 μm-thick transverse sections at the mid–papillary muscle level and fixation in 4% formalin (zinc-buffered fixative was used for ICAM-1 and VCAM-1 detection). After fixation, tissues were embedded in paraffin. As previously described, myocardial cross sections were oriented on a defined grid. An image analysis system (Optimus Corp) was used to calibrate type-specific cell populations sections evaluated per heart.

Quantification of End Points on Histology

Hearts were prepared for histological examination by cutting serial 5 μm-thick transverse sections at the mid–papillary muscle level and fixation in 4% formalin (zinc-buffered fixative was used for ICAM-1 and VCAM-1 detection). After fixation, tissues were embedded in paraffin. As previously described, myocardial cross sections were oriented on a defined grid. An image analysis system (Optimus Corp) was used to calibrate type-specific cell populations 18 standard fields of myocardium for each cross section, viewed at 200-fold magnification (400-fold magnification for NF-κB quantification). Data presented for each heart represent the mean values of the 18 fields per cross section averaged over the 5 to 6 such sections evaluated per heart.

The prevalences of NF-κB activation and ICAM-1 and VCAM-1 expression in endothelial cells were assessed quantitatively within myocardial specimens by counting the vessels that had any positive endothelial staining divided by the total number of vascular profiles (defined by platelet and endothelial cell adhesion molecule-1, CD31, staining). Vessels in which immunostaining was present but not circumferential were counted as positive for this analysis. NF-κB activation of cardiac myocytes was calculated as the percentage of myocytes positively stained for activated p65 divided by the total number of myocyte nuclei. NF-κB activation and ICAM-1 expression on infiltrating host leukocytes were not evaluated because treatment was directed only to the donor organ endothelium. T-lymphocyte infiltration was quantified by computerized image analysis of immunocytochemistry specimens; neutrophils were counted manually on the same grid system; leukocyte data are presented as the mean number of cells per field.

Immunocytochemistry

Immunocytochemistry was used to identify infiltrating T lymphocytes, endothelial VCAM-1 and ICAM-1 expression, and NF-κB activation. As previously, the antibodies used were directed against rabbit T lymphocytes, monoclonal antibody (mAb) Thi 188 (1:500 dilution, American Type Culture Collection), rabbit VICM-1, mAb RB 1/9 (1:100 dilution), and rabbit ICAM-1, mAb RB 2/3 (1:500 dilution) (both gifts from Dr Myron Cybulsky). Activated NF-κB was detected with a monoclonal antibody to an epitope on the p65 subunit (1:50 dilution; anti-NF-κB–p65, Roche Molecular Biologicals) that is exposed only on nuclear translocation and thus binds only to the active, nuclear form of NF-κB.

Briefly, after deparaffinization and dehydration, all sections were incubated in methanol/3% hydrogen peroxide 1:1 for 10 minutes to block endogenous peroxidase, then in blocking solution (1% BSA/PBS, 1% horse serum, and 0.1% Triton X-100) for 30 minutes, followed...
by incubation with primary antibodies for 1 hour at room temperature. After washing with PBS, all sections were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories) for 30 minutes, washed, then treated with avidin-peroxidase (ABC Elite kit, Vector Laboratories) for 30 minutes. The reaction was developed with DAB substrate (Vector Laboratories). Finally, sections were counterstained with hematoxylin to quantify the total number of nuclei per field as the denominator for the calculations above.

Antigen unmasking was required for NF-κB detection, accomplished after alcohol dehydration by placing sections in a 0.1 mol/L citrate buffer bath (pH 6.0), which was then heated for 10 minutes in a microwave oven.

**Neutrophils**

Neutrophils were identified by esterase staining (naphthol AS-D chloroacetate esterase procedure, Sigma).

**Apoptosis**

Paraffin sections from 2 eNOS-transfected and 1 control D/W-treated donor hearts were examined for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (In Situ Cell Death Kit, Roche Molecular Biochemicals) to label DNA strand breaks. Apoptotic cells in vascular profiles and cardiac myocytes were evaluated separately.

**Statistical Analysis**

Continuous variables are presented as the mean±SEM; comparisons between groups were performed with Student’s t test; differences were considered to be statistically significant at a value of P<0.05.

**Results**

**Reporter Gene Pilots**

In 7-day pilots, mean CAT enzyme activities in homogenized right and left ventricular myocardial specimens from the liposome-CAT–treated, transplanted donor hearts were 5- to 7-fold higher than the baseline “negative” readings in native hearts. Consistent with vascular endothelial cells as the primary transfection targets, reporter gene expression was

---

**Figure 4.** Adhesion molecule expression and leukocyte infiltration in transplanted donor hearts. Histological sections of transplanted donor hearts at 24 hours after transfection by liposomes complexed to eNOS on left (a, c, e, g) or pUC control plasmids on right (b, d, f, h). For immunocytochemistry panels (c through h), positive immunoreactivity is seen as brown (DAB-positive) staining; nuclei are counterstained blue with hematoxylin. Bar=100 μm. a and b, Neutrophil infiltration. Marked infiltration in the control heart is compared with minimal infiltration in the eNOS-transfected heart. Hematoxylin and eosin stain. Formalin fixation. c and d, T-lymphocyte infiltration. T-lymphocyte infiltration is noticeably less in the eNOS-transfected heart. Formalin fixation. e and f, ICAM-1 immunoreactivity. In the eNOS-treated heart (e), ICAM-1 is not visualized on transfected endothelium but is still seen, to a lesser extent, on infiltrating host leukocytes that were not transfected. ICAM-1 is seen on both vascular endothelium and infiltrating leukocytes in the pUC-transfected control heart (f). Zinc fixation. g and h, VCAM-1 immunoreactivity on microvascular endothelium. VCAM-1 is not expressed on endothelium in this eNOS-treated specimen (g) but is present in the control heart (h). Zinc fixation.
reliably persistent in all aortic (coronary ostial) specimens at 7 days but 10-fold lower than in similar specimens examined at 24 hours. At 7 days, mean CAT activities were 36 ± 4.8 vs 4.7 ± 1.1×10^6 cpm/mg in the treated and native aortic specimens, respectively (P<0.001).

Nitrite Production
We observed significantly higher production of nitrite in aortic (coronary ostia) and coronary sinus specimens obtained from eNOS-transfected hearts than from control hearts treated with liposome-plasmid complexes without a functional gene or from untransplanted native hearts (P<0.01, Figure 1). Given the rapid first-pass adherence of cationic liposomes to endothelium, we observed higher levels of nitrite production near the delivery site in the aorta than in the coronary sinus (414 ± 66 versus 149 ± 25 nmol · L nitrite · mg tissue · 1, P<0.01) in the eNOS-transfected hearts. Furthermore, in eNOS-transfected hearts, nitrite production increased 4-fold after calcium ionophore treatment. This calcium dependence is strong evidence that the majority of NO was produced by eNOS expressed from the transfected gene. In these vascular specimens, there were no other significant differences in endogenous calcium-dependent or -independent nitrite production between liposome-transfected control donor hearts and native hearts.

NF-κB Activation
The prevalence of NF-κB activation (NF-κB nuclear localization) in intramyocardial coronary endothelial cells was significantly reduced in eNOS-transfected myocardium (9 ± 1 cells/field) compared with pUC-transfected myocardium and to diluent (D5W)-treated controls (27 ± 3% versus 87 ± 3% and 70 ± 4%, respectively, P<0.01) (Figures 2 and 3a). NF-κB activation in eNOS-treated, transplanted hearts was still higher than in untransplanted native hearts (P<0.05). Among cardiac myocytes, the prevalence of NF-κB activation was 65% less in the eNOS-transfected hearts than in control donor hearts (19 ± 2% versus 55 ± 3% and 55 ± 4%, respectively, P<0.01) (Figure 3b).

Endothelial ICAM-1 and VCAM-1 Expression
The percentage of intramyocardial vascular profiles with endothelial expression of ICAM-1 and VCAM-1 expression were both significantly reduced in eNOS-treated hearts compared with control hearts treated with liposome-pUC plasmid complexes and those treated with the diluent, D5W, alone (ICAM-1: 46 ± 5% versus 92 ± 1% and 80 ± 3%, respectively; VCAM-1: 47 ± 14% versus 92 ± 1% and 91 ± 6%) (Figures 4e through 4h and 5). Adhesion molecule expression in transplanted eNOS-transfected hearts, however, was still higher than in untransplanted native hearts (endothelial ICAM-1, 2 ± 0.5%; endothelial VCAM-1, 2 ± 1%, respectively).

Leukocyte Infiltration at 24 Hours
The number of neutrophils per field was 50% less in eNOS-transfected myocardium (9 ± 1 cells/field) compared with pUC-transfected myocardium (18 ± 1 cells/field, P<0.01) and control myocardium treated with diluent only (16 ± 4.3 cells/field, P<0.05) (Figures 4a and 4b and 6a).

Figure 5. ICAM-1 and VCAM-1 expression in coronary microvasculature. Endothelial expression of ICAM-1 (a) and VCAM-1 (b) at 24 hours in transplanted donor hearts treated with liposomes complexed to eNOS or pUC control plasmids, hearts treated with D5W diluent only, and native hearts. Bars represent mean±SEM. **P<0.01.

Figure 6. Leukocyte infiltration in transplanted donor hearts. Neutrophil (a) and T-lymphocyte (b) infiltration at 24 hours in transplanted donor hearts treated with liposomes complexed to eNOS or pUC control plasmids, hearts treated with D5W diluent only, and native hearts. Data represent mean±SEM. *P<0.05; **P<0.01.
A significant reduction in T-lymphocyte infiltration was also demonstrated in eNOS-transfected hearts compared with hearts transfected with the pUC control plasmid (89 ± 16 versus 172 ± 21 cells/field, \( P < 0.01 \)) as well as hearts treated with the diluent, D/W, alone (155 ± 12 cells/field, \( P < 0.01 \)) (Figures 4c and 4d and 6b).

**Apoptosis**
The prevalence of apoptosis was examined to ascertain whether increased NO production would cause apoptosis. In both the eNOS-transfected and control donor hearts, <1% of vascular endothelial and smooth muscle cells had evidence of apoptosis by TUNEL at 24 hours after transplantation. Among cardiac myocytes, only 4% of myocytes in the eNOS-transfected hearts had positive TUNEL staining, compared with 29% in the untreated control donor hearts.

**Histology at 7 Days**
At 7 days after transplantation, 2 eNOS-transfected transplanted hearts evidenced markedly less vascular rejection than untreated control hearts (19% versus 70% of arterial profiles with vascular rejection, respectively; Figure 7). eNOS-transfected hearts also had fewer T lymphocytes (mean 161 versus 288 cells/field) and macrophages (mean 15 versus 27 cells/field) present at this time point. Only rare neutrophils were seen in either group.

**Graft Survival**
Without immunosuppression, donor hearts transfected with a single dose of liposome-eNOS complexes before transplantation survived significantly longer (13.5 ± 1.3 days) than control donor hearts without liposome-gene transfection (9.3 ± 1.8 days, \( P < 0.05 \)) (Figure 8).

**Discussion**
Adhesion of host leukocytes to donor endothelium is the first step in ischemia-reperfusion injury as well as in graft cellular rejection. We have previously shown that antibody blockade of adhesion molecule receptors on recipient leukocytes can markedly reduce both neutrophil and T-lymphocyte infiltration of the transplanted donor heart during ischemia-reperfusion injury \(^5\) and early graft rejection \(^10\) in this model. Host leukocyte alteration, however, like other existing immunosuppression, impairs host defenses against infection. In these studies, we investigate whether the same end point of adhesion molecule blockade could be achieved by modification of the donor coronary endothelial adhesion ligands instead of the recipient leukocyte receptors.

These experiments demonstrate that liposome gene delivery of eNOS, even given current transfection inefficiencies, was sufficient to reduce activation not only of the transfected endothelial cells but also of the surrounding myocytes. Also, by using perfusion delivery techniques and endothelial cell transfection, which may be more physiological, we did not see the myocardial apoptosis recently reported with a gene therapy strategy that employed direct myocardial injection of other eNOS-liposome vectors. \(^11\)

Previous studies have demonstrated that myocardial ischemia-reperfusion injury is exacerbated in the absence of eNOS \(^2\) and that inhaled NO \(^3\) or infusion of NO donors will ameliorate ischemia-reperfusion injury in several models. \(^14\)–\(^16\) Organ-
specific gene therapy approaches may make it possible to generate therapeutic local levels of NO at target tissue sites without incurring systemic side effects. Several previous in vivo studies, using both liposome and adenoviral vectors, have used arterial gene transfer of eNOS to inhibit neointimal formation in nontransplant settings. Known beneficial effects of eNOS on coronary vasodilatation, platelet function, endothelial cell survival and physiology, vascular wall activation, and cell-regulatory events may be operative in the amelioration of early coronary endothelial activation and later vascular rejection in this transplant model.

The fact that vascular nitrite production was increased only in eNOS-transfected specimens, with little contribution of endogenous nitrite production, supports the premise that gene transfer was responsible for the observed results. Any concomitant detrimental (or even cardioprotective) effects of myocyte and/or macrophage iNOS were controlled for in the liposome-transfected, transplanted hearts without functional genes.

Although not statistically significant, it was of interest that higher mean prevalences of NF-κB activation, adhesion molecule expression, and leukocyte infiltration were seen in the control hearts transfected with the liposome-pUC plasmid complexes than in the hearts treated with DMSO alone. This might represent an activation phenomenon induced either by the liposomes or, more likely, by the bacterial plasmid DNA as a result of unmethylated CpG motifs, which can contribute to immune activation, immediate NF-κB activation, and inflammatory gene induction. If so, the NO produced by the transduced eNOS presumably also downregulated this mild activation induced by the control liposome-plasmid complexes, an important safety point for clinical applications.

In summary, these studies demonstrate that intravascular liposome delivery of the eNOS gene to donor hearts leads to measurable endothelial expression of NO after transplantation, reductions in early endothelial activation and leukocyte infiltration, and extended graft survival without immunosuppression. This simple intraoperative technique may herald the clinical application of gene therapy as adjunctive therapy in the transplantation of hearts and other solid organs as well as in routine cardiac surgery outside the realm of transplantation.

Acknowledgments

This work was supported by the American Heart Association, grant 9650559N. The authors would like to acknowledge Dr Jeanette Ennis for reviewing the manuscript and the Valentis Corporation for providing the liposome complexes used in this study.

References

Liposome-Mediated Gene Transfection of Endothelial Nitric Oxide Synthase Reduces Endothelial Activation and Leukocyte Infiltration in Transplanted Hearts

Akiko Iwata, Sadahiro Sai, Yoshio Nitta, Megan Chen, Ricarda de Fries-Hallstrand, Joy Dalesandro, Robert Thomas and Margaret D. Allen

_Circulation_. 2001;103:2753-2759
doi: 10.1161/01.CIR.103.22.2753

_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/103/22/2753