Antiangiogenesis Mediates Cisplatin-Induced Peripheral Neuropathy
Attenuation or Reversal by Local Vascular Endothelial Growth Factor Gene Therapy Without Augmenting Tumor Growth

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Background—Toxic neuropathies induced by cisplatin and other chemotherapeutic agents are important clinical problems because of their high incidence, their lack of effective treatment, and the fact that neuropathy represents a dose-limiting factor for these therapies. The pathogenic basis for toxic neuropathies induced by chemotherapeutic agents has not been completely elucidated.

Methods and Results—We investigated the hypothesis that experimental toxic neuropathy results from an antiangiogenic effect of these drugs, resulting in destruction of the vasa nervorum, and accordingly that the neuropathy could be prevented or reversed by locally administered VEGF gene transfer without augmenting tumor growth. In an animal model of cisplatin-induced neuropathy, nerve blood flow was markedly attenuated, and there was a profound reduction in the number of vasa nervorum associated with marked endothelial cell apoptosis, resulting in a severe peripheral neuropathy with focal axonal degeneration characteristic of ischemic neuropathy. After intramuscular gene transfer of naked plasmid DNA encoding VEGF-1 in animals with an established neuropathy, vascularity and blood flow returned to levels similar to those of control rats, peripheral nerve function was restored, and histological nerve architecture was normalized. Gene therapy administered in parallel with cisplatin chemotherapy completely attenuated endothelial cell apoptosis and inhibited destruction of nerve vasculature, deterioration of nerve function, and axonal degeneration. In a rat tumor model, VEGF gene transfer administered locally did not alter tumor growth or vascularity.

Conclusions—These findings implicate microvascular damage as the basis for toxic neuropathy induced by cisplatin and suggest that local angiogenic gene therapy may constitute a novel prevention or treatment for this disorder without augmenting tumor growth or vascularization. (Circulation. 2005;111:2662-2670.)

Key Words: angiogenesis ■ endothelium ■ gene therapy ■ vasa nervorum

Most chemotherapeutic drugs known to induce a toxic neuropathy—ie, cisplatin, thalidomide, and taxol—are, in addition to their direct antimitotic effect, potent antiangiogenic agents.1-3 We therefore hypothesized that toxic neuropathies induced by these agents might be caused by destruction of the nerve blood supply (vasa nervorum) and might therefore benefit from factors capable of stabilizing or regenerating the microvasculature of the peripheral nerves.

We report here that cisplatin exerts antiangiogenic activity in vitro and in vivo. Cisplatin-induced neuropathy in rats was characterized by marked endothelial cell (EC) apoptosis within the vasa nervorum and reduction in nerve blood perfusion and in the density of vasa nervorum as shown by laser Doppler perfusion imaging (LDPI) and by EC-specific BS1 lectin staining, respectively. VEGF gene therapy (GTx) restored nerve blood supply, electrophysiological nerve function, and histological nerve architecture. Prophylactically, locally administered VEGF GTx completely inhibited cisplatin-induced EC apoptosis in the vasa nervorum, prevented the onset of neuropathy, but did not augment tumor growth.
Methods

Cell Culture

Human umbilical vein ECs (HUVECs) were isolated and cultured in 6- or 24-well plates and allowed to attach overnight. Cells in 6-well plates were starved in M199/1% BSA (Sigma), treated with 20 μmol/L cisplatin (Bristol-Myers Squibb) for 18 hours, and processed for Western blotting. The 24-well plates were treated with different concentrations of cisplatin for 48 hours. Supernatant was collected for lactate dehydrogenase determination (Promega Cytotoxic Assay); cells were fixed for paraformaldehyde (PFA) and stained with DAPI (4′,6-diamidino phenylidole, 1 μg/mL PBS for 20 minutes).

In some experiments, cells were starved and incubated with 20 μmol/L cisplatin with or without 100 ng VEGF (Sigma), 1 μmol/L LY294002 (Sigma), or 1 μmol/L PD98059 (Sigma) for 24 hours and stained with DAPI.

In another set of experiments, HUVECs were transfected with an adenovirus expressing a constitutively active form of Akt (adeno-myrAkt) using a multiplicity of infection of 50. Cells were then starved for 18 hours with or without 20 μmol/L cisplatin and subjected to DAPI staining.

Cells were examined by fluorescence microscopy, and 3 randomly selected fields per well were photographed. Total cell number and apoptotic cells, characterized by condensation and fragmentation of the nucleus, were counted. Experiments were performed in triplicate.

Western Blotting

Antibodies were purchased as follows: phospho-Akt (Ser 473) and Akt from Cell Signaling, anti-ACTIVE MAPK and anti-ERK 1/2 from Promega, and actin from Santa Cruz. Cells were lysed, lysates were processed, and Western blotting was performed as suggested by the manufacturer. Bands were visualized by ECL staining for 1 minute (Amersham Pharmacia Biotech).

Animal Models

All protocols were approved by the St Elizabeth’s Medical Center Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to the identity of the treatment administered.

Mouse Cornea Neovascularization Model

Because of conflicting reports in the literature about the in vivo antiangiogenic effect of cisplatin, mouse corneal neovascularization assay was performed as described.

Figure 1. Cisplatin inhibits in vivo angiogenesis. Corneal micro-pocket assay. VEGF containing pellets (b, f) induced robust angiogenic response vs solvent-alone pellet (a, e). Systemic administration of cisplatin (c, g) or taxol (known antiangiogenic agent, included as positive control; d, h) significantly reduced VEGF-induced neovascularization. Area of neovascularization, calculated as described using vessel length and circumference of neovascularization, was 1.82±0.14 mm² for VEGF alone (n=4), 0.67±0.03 mm² for VEGF plus cisplatin (n=4), and 0.71±0.09 mm² for VEGF plus taxol (n=3). P<0.001, cisplatin- and taxol-treated animals vs VEGF alone.

Figure 2. a, VEGF GTx restores nerve function in cisplatin-induced neuropathy. Both MCV and SCV (right) decreased significantly after cisplatin therapy vs baseline (point a). VEGF-treated animals (red) showed significant improvement in SCV and MCV after VEGF vs solvent-alone (point b). b, Saline injections did not result in significant improvement in nerve function (SCV: 6 weeks after GTx: VEGF, 46.5 m/s; saline, 40.8 m/s; 12 weeks: VEGF, 47.3 m/s; saline, 39.6 m/s; MCV: 3 weeks after GTx: VEGF, 44.5 m/s; saline, 39.1 m/s; 6 weeks: VEGF, 46.8 m/s; saline, 41.6 m/s; 12 weeks: VEGF, 49.2 m/s; saline, 41.9 m/s; P<0.05; n=16 for saline, n=18 for VEGF). *P<0.05, VEGF GTx vs saline. b, VEGF GTx prevents cisplatin-induced neuropathy. Injections of VEGF plasmid 48 hours before cisplatin applications at weeks 1, 3, 5, 7, and 9 inhibit decrease in MCV and SCV after 9 weeks of cisplatin. P=NS, baseline vs 9 weeks after treatment; *P<0.05, VEGF plus cisplatin (n=10) vs cisplatin alone (n=34).

Rat Cisplatin-Induced Neuropathy

Male Sprague-Dawley rats (retired breeders, Charles River Laboratories, Wilmington, Mass) weighing 550 to 650 g were used. Rats were fed standard laboratory rodent chow and water ad libitum and housed individually.

Induction of Cisplatin-Induced Neuropathy

Forty-seven rats were treated by intraperitoneal injections of 2 mg/kg cisplatin (Bristol-Myers Squibb) as described by other investigators once weekly for 9 weeks under anesthesia. Concomitantly, 10 mL sterile saline was injected subcutaneously to ensure sufficient hydration to prevent renal damage. Age- and weight-matched rats injected with saline were used as control animals.

Gene Transfer With phVEGF165

GTx was accomplished with plasmid DNA expressing human VEGF165 (phVEGF165). Two groups were studied. In the first group (n=34), VEGF was given after 9 weeks of cisplatin. In the second (n=10), VEGF was given concomitantly with the cisplatin treatment (48 hours before cisplatin injections at weeks 1, 3, 5, 7, and 9) in an attempt to study the potential prophylactic effect of GTx on the neuropathy. Animals received intramuscular injections of 200 μg phVEGF165 as described. Placebo-injected animals received saline.
Tumor Model
Cisplatin-sensitive Walker 256 tumors (courtesy NCI–Frederick Cancer DCT Tumor Repository) were engrafted into the flanks of Sprague-Dawley rats. Injection of cisplatin into rats immediately after tumor inoculation inhibited tumor engraftment. Therefore, VEGF plasmid was injected 1 and 3 weeks after tumor inoculation (plasmid was injected intramuscularly into hindlimbs along the sciatic nerves as described above), and cisplatin was injected at weeks 2, 3, and 4 (the same doses for VEGF and cisplatin as in the neuropathy studies were used). Tumor volume (cm³) was measured weekly. After 4 weeks, tumor samples were excised and fixed in 4% PFA for determination of vessel density. Sections were stained with biotinylated isolectin B4 (Vector Labs) and incubated with peroxidase-labeled streptavidine (Signet Labs) and 3-amino-9-ethylcarbazole (AEC, Vector Labs). Ten different sections per tumor were counted by an investigator blinded to treatment assignment.

Neurophysiological Measurements

Electrophysiology
Sciatric nerve motor and sensory nerve conduction velocities (NCV) were measured as described previously. During measurements, rats were kept on a heating pad, and the temperature of the animals was kept constant at 37°C. Body temperature was monitored by repeated measurements.

In Vivo Assessment of Nerve Perfusion and Vascularity

LDPI of Vasa Nervorum Blood Flow
Perfusion of sciatric vasa nervorum was measured with an LDPI system (Moor Instruments) as described previously in detail.

Fluorescent Imaging of Vasa Nervorum
Vascularity of sciatric nerves was assessed by in situ fluorescent staining using the EC-specific marker BS-1 lectin conjugated to FITC (Vector Laboratories) as described previously.

Determination of In Vivo EC Apoptosis in Sciatic Nerves
Cisplatin-induced apoptosis of ECs in vasa nervorum was determined with TUNEL staining (Roche Diagnostics) of PFA-fixed sciatric nerve cross sections as described by the manufacturer. Sections from control rats and rats treated with cisplatin or cisplatin concomitantly injected with VEGF plasmid were used. For double labeling of apoptotic ECs in the vasa nervorum, double immunofluorescence for TUNEL staining (FITC labeled) and CD31 antibody (Pharmingen) was performed. In a subset of animals, nerves were harvested after 2 weeks to document the extent of early EC apoptosis.

Nerve Histology
For morphological examination, sciatric nerves were carefully dissected and immersion fixed with 4% glutaraldehyde overnight. Nerves were subsequently postfixed with 1% osmium tetroxide for 2 hours, dehydrated, and embedded in epoxy resin. Semithin (1 μm) sections were cut with a glass knife and stained with 1% methylene blue.

Statistical Analysis
All results are expressed as mean±SEM. Statistical comparisons between groups were performed by ANOVA. Values of P<0.05 were considered to denote statistical significance. In case of nonnormal distribution, nonparametric tests were used.

Results

Corneal Neovascularization Assay
Systemic cisplatin (n=4) and taxol (a known antiangiogenic agent included as a control) significantly reduced VEGF-induced corneal neovascularization (Figure 1), revealing significant inhibition of angiogenesis by systemic cisplatin, comparable to the effect of taxol, a known antiangiogenic agent.

Nerve Electrophysiology
Cisplatin administration resulted in a classic pattern of motor and sensory neuropathy as previously described. Both motor NCV (MCV) and sensory NCV (SCV) were stable over the time of the study period in control animals (data not shown). Cisplatin treatment led to a significant reduction in MCV (39.4 m/s; P<0.05 versus baseline) and SCV (37.2 m/s; P<0.05 versus baseline) 5 days after the last cisplatin injection.

At the end of cisplatin therapy, rats were randomly selected to receive VEGF-1 GTx or placebo (saline) injection. NCV 3, 6, and 12 weeks after VEGF GTx showed an increase in SCV 6 at 12 weeks after GTx (Figure 2a) and an increase in MCV 3, 6, and 12 weeks after GTx, yielding a significant difference between the NCV of VEGF- and saline-treated groups and between NCV before and after VEGF therapy. NCV did not increase significantly after saline injection.
A subgroup of rats received VEGF GTx before cisplatin injections to evaluate the effect of VEGF GTx pretreatment on cisplatin-induced neuropathy. As shown in Figure 2b, VEGF GTx effectively prevented cisplatin-induced neuropathy (MCV, 39.4 m/s; SCV, 37.2 m/s; both $P<0.05$ versus VEGF pretreatment; Figure 2b).

Sciatic Nerve Histology

In cisplatin-treated rats, signs of focal axonal degeneration (especially below the perineurium), loss of myelinated fibers, myelin phagocytosis by macrophages, and areas of fibrosis were observed (Figure 3b). In animals receiving VEGF treatment after cisplatin therapy (Figure 3c) and in rats that received VEGF in parallel with cisplatin (Figure 3d), axonal architecture was restored or preserved, comparable to the histology of a normal nerve (Figure 3a).

LDPI of Sciatic Nerve Blood Flow

Sciatic nerve imaging (Figure 4A) showed reduced nerve blood flow in cisplatin-treated animals, whereas VEGF GTx after the induction of cisplatin-induced damage restored nerve blood flow (LDPI units: controls, 1181.2±101.5, n=10; cisplatin, 716.3±97.6, n=7; cisplatin plus VEGF, 1112.8±63, n=7; controls versus cisplatin, $P<0.01$; cisplatin plus VEGF versus cisplatin, $P<0.01$; cisplatin plus VEGF versus controls, $P=NS$). Rats receiving VEGF GTx simultaneously with cisplatin did not show a decrease in nerve blood flow ($P=NS$ versus controls). These data indicate that VEGF GTx can restore or preserve sciatic nerve perfusion in the setting of cisplatin-induced peripheral neuropathy.

In Vivo Staining of Vasa Nervorum by BS-1 Lectin Perfusion

Whole-mount FITC-conjugated BS-1 lectin staining (Figure 4B, top) showed markedly reduced vasa nervorum in cisplatin-treated, saline-injected animals, whereas VEGF gene transfer (Figure 4c and 4g) performed after cisplatin treatment restored the nerve vasculature, as judged by gross...
observation and quantification (bottom). VEGF pretreatment before cisplatin inhibited the loss of vasa nervorum (Figure 4d and 4h).

### VEGF GTx Does Not Accelerate Tumor Growth

We performed an additional series of experiments in a tumor model to determine whether VEGF GTx administered at a remote site would augment growth or vascularity of an existing tumor. We selected an established, cisplatin-sensitive tumor cell line (Walker 256), inoculated rats with tumor cells, and allowed a solid tumor to develop for 2 weeks before initiating cisplatin treatment. VEGF GTx identical to that performed in the above experiments or control saline administration was started 1 week after tumor inoculation and 1 week before initiation of chemotherapy. This strategy was designed to provide the maximum opportunity for VEGF to augment tumor vascularity or growth, yet no difference was observed between VEGF GTx- and saline-treated rats in tumor size (Figure 5a) or tumor capillary density (Figure 5b through 5d).

### VEGF Inhibits Cisplatin-Induced Apoptosis of ECs

To determine whether the attenuation of the nerve vasculature was the result of direct effects of cisplatin on ECs, in vitro studies were performed to document the effect of cisplatin on EC viability and cell number and to determine whether VEGF could alter the effect of cisplatin on EC.

As shown in Figure 6A, cisplatin increased the number of pyknotic EC nuclei, consistent with EC apoptosis, and reduced EC counts (to 43% of controls at 10 μmol/L cisplatin) in a dose-dependent manner. LDH levels in cell supernatants were similar to those of controls at all doses, excluding cell necrosis resulting from drug cytotoxicity (data not shown).

Figure 6b shows the effect of VEGF on cisplatin-induced EC apoptosis. Cells were serum starved and treated with 20 μmol/L cisplatin alone or in combination with 100 ng VEGF for 24 hours. Addition of VEGF to cisplatin-treated cells resulted in significantly reduced EC apoptosis and significantly increased cell numbers compared with cells treated with cisplatin alone. These data indicate that cisplatin has a direct apoptotic effect on ECs that is inhibited by EC survival and the growth factor VEGF, consistent with the in vivo studies.

### Cisplatin Induces Apoptosis of ECs of the Sciatic Nerve Vasa Nervorum: Prevention by VEGF GTx

As shown in Figure 7A, TUNEL-positive cells (arrowheads) are abundant in the sciatic nerves of cisplatin-treated rats (Figure 7A) compared with controls, with many of the positive cells lining the walls of endoneurial vessels (arrows). VEGF GTx results in a significant decrease in the apparent rate of apoptosis. Double immunofluorescent staining for TUNEL and CD31 immunoreactivity reveals that most TUNEL-positive cells in the sciatic nerves of cisplatin-treated rats are ECs (Figure 7A).

To provide further supportive evidence that EC damage was a primary occurrence in the advent of cisplatin-induced neuropathy, we harvested sciatic nerves only 2 weeks after the onset of drug administration, well before the onset of nerve dysfunction. As shown in Figure 7B, there is abundant evidence of EC apoptosis, defined by the colocalization of TUNEL-positive cells with cells expressing the EC marker isolectin B4. Although correlative, these data reveal a temporal relationship between early EC damage and subsequent neurological dysfunction that suggests a causative role for destruction of the vasa in the development of this neuropathy.

### Cisplatin Inhibits PI3-Kinase/Akt Pathway and Activates MAPK Pathway in ECs

Having established the induction of EC apoptosis by cisplatin in vitro and in vivo, we next sought to investigate candidate signaling pathways mediating this effect.

Figure 8a shows Western blot analysis of cultured, serum-starved HUVECs treated with cisplatin. As demonstrated with specific antibodies against the active, phosphorylated form of Akt, treatment with cisplatin (20 μmol/L) for 18 hours decreased activated Akt and total (phosphorylated and unphosphorylated) Akt. Phosphorylated (activated) ERK 1/2
shows marked activation after 18 hours of stimulation. Total ERK was not changed.

As shown in Figure 8b, addition of the PI3-kinase inhibitor LY294002 to cisplatin-treated cells increased EC apoptosis compared with cisplatin alone. In contrast, inhibition of MAPK signaling by PD98059 together with cisplatin did not further increase apoptosis compared with cisplatin alone. Because Akt is a known survival pathway in EC, the downregulation of the active, phosphorylated form of Akt by cisplatin suggested that impairment of Akt might be responsible for cisplatin-induced EC apoptosis. The importance of the PI3-kinase/Akt signal transduction pathway was further suggested by transfection of HUVECs with an adenovirus expressing constitutively active myrAkt. As shown in Figure 8c, overexpression of myrAkt inhibited cisplatin-induced EC apoptosis and the associated decrease in total cell number. Together, these data imply that cisplatin-mediated neuropathy may result from a direct effect on EC of the vasa nervorum by reducing Akt activity, resulting in EC apoptosis and attenuation of the nerve blood supply with resultant nerve destruction and malfunction.

**Discussion**

Previous studies in our laboratory have demonstrated that GTx with the endothelial mitogen VEGF is able to restore nerve function in ischemic and diabetic neuropathy. In animal models of diabetic neuropathy, the nerve blood supply through vasa nervorum was markedly reduced, a pathological condition also observed in human diabetic neuropathy. VEGF GTx in these settings normalized the vasa nervorum and nerve perfusion, as well as electrophysiological nerve function. Additionally, VEGF-1 GTx was able to improve ischemic neuropathy in some patients, including diabetics, treated for critical limb ischemia.

Here, we show that the neuropathy induced by certain chemotherapeutic agents is associated with apoptosis of ECs comprising the vasa nervorum, resulting in pathological and physiological changes consistent with ischemic neuropathy. Moreover, our data indicate that the neuroprotective and regenerative capabilities of angiogenic GTx may also extend to chemotherapy-induced neuropathies. Finally, these studies also reveal that local plasmid-mediated angiogenic GTx can achieve therapeutic effects in the setting of chemotherapy-induced neuropathy without enhancing tumor growth or vascularity.

The mechanism of cisplatin neuropathy is still under considerable discussion. It has previously been shown to induce apoptosis of sensory neurons in the dorsal root ganglia, and this damage is considered by some to be the main pathogenic mechanism in cisplatin-induced neuropathy. However, long-term follow-up of patients with cisplatin neuropathy has shown improvement of nerve function over time, an unlikely scenario if primary neuronal damage were responsible. Additionally, high concentrations of platin were found in dorsal root ganglia and in the peripheral nerves in an autopsy study, demonstrating that...
cisplatin might also damage the nerve itself.\textsuperscript{20} Accordingly, we considered the possibility that the vasa nervorum, which exhibits a regenerative capacity,\textsuperscript{10} might be involved. A vascular pathogenesis is attractive because most chemotherapeutic agents that induce neuropathy also show antiangiogenic activity in addition to their antimitotic properties.\textsuperscript{1–3}

One key finding in our study was the marked reduction of nerve blood supply in cisplatin-induced neuropathy as shown by LDPI and in vivo BS1 lectin staining. Preliminary data on taxol- and thalidomide-induced neuropathies (R. Kirchmair and J.M. Isner, unpublished observations) also show a reduction in nerve perfusion, supporting the hypothesis that antiangiogenic chemotherapeutic agents cause a neuropathy at least in part by a vascular mechanism.

To further elucidate the role of the microvasculature in cisplatin-induced neuropathy, we used the strategy of intramuscular VEGF-1 gene transfer in proximity to the sciatic nerve\textsuperscript{10} to restore or to preserve nerve vasculature, nerve function, and nerve histology. VEGF GTx successfully prevented the loss of vasa nervorum, development of neuropathy, and axonal degeneration when applied simultaneously with cisplatin (prophylactic approach). Furthermore, when VEGF-1 GTx was instituted after neuropathy was established (therapeutic approach), it fully restored nerve blood supply, physiological nerve function, and the histological architecture of the nerve. An alternative or complementary mechanism for the effect of VEGF could include a direct neurotrophic effect, as we have noted previously.\textsuperscript{11}

Previous reports have demonstrated that cisplatin inhibits proliferation of ECs in vitro.\textsuperscript{1,6} We demonstrate here that cisplatin directly induces apoptosis of ECs in vitro and in vivo, associated with degeneration of the vasa nervorum, destruction of nerve architecture, and loss of nerve function. All of these events are reversed or prevented in vivo by VEGF.

These data also highlight a gap in our understanding of angiogenesis: the tissue/organ specific angiogenic response to systemic stimuli. The most ubiquitous example of this phenomenon may be diabetes, in which both excess angiogenesis (retina) and deficient angiogenesis (myocardium, peripheral nerve, lower extremity) appear to be driven by the same disease process. In cancer, the tumor develops an auxiliary blood supply, seemingly without impact on angiogenesis in other organs. Here, we show disrupted angiogenesis in the vasa nervorum induced by cisplatin and restoration after VEGF GTx without apparent effects on other tissues. A corollary question involves the effect of VEGF GTx on the vasa nervorum versus other vasculature. This question was, in part, the motivation for our tumor studies. Tumor-bearing rats were randomized to receive VEGF GTx or saline injections with cisplatin started the following week, plus readministration of VEGF GTx (versus saline) at 2-week intervals and cisplatin at weekly intervals. These studies revealed that the growth rate (cisplatin failed to eradicate these established tumors) and vascularity of the tumors in VEGF and control rats were indistinguishable. This paradox—an apparent role of VEGF in the growth of certain tumors and a lack of effect of administered angiogenic cytokines on tumor growth—suggests that VEGF is necessary but not sufficient for carcinogenesis. Although our results of VEGF GTx on
cisplatin neuropathy are encouraging, this question will need to be addressed cautiously in clinical application in humans.

In conclusion, we demonstrate that cisplatin-induced neuropathy is associated with the induction of EC apoptosis and destruction of the vasa nervorum and is reversed or inhibited by GTx with the angiogenic cytokine VEGF. These data suggest a potential strategy to preserve nerve function in patients undergoing chemotherapy with these agents that may allow more effective dosing by the prevention of this disabling toxicity.

Acknowledgments
This project was supported in part by NIH grants (HL-53354, HL-57516, HL-60911, HL-63414, HL-63695, AG-16332, and HL-66957) and the Shaughnessy Center for Clinical Genetics. Dr Kirchmair is the recipient of a fellowship from the Max-Kade Foundation. We would like to dedicate this study to the late Jeffrey M. Isner, who initiated this work and was a great teacher and a pioneer in the field of therapeutic angiogenesis. We also thank Dr Alan Peters and Claire Folger from Boston University for technical assistance in nerve histology. We gratefully acknowledge the expert secretarial assistance of Mickey Neely and Deirdre Costello.

References


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_Circulation_. 2005;111:2662-2670; originally published online May 16, 2005; doi: 10.1161/CIRCULATIONAHA.104.470849

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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