Local Gene Transfer of phVEGF-2 Plasmid by Gene-Eluting Stents
An Alternative Strategy for Inhibition of Restenosis

Dirk H. Walter, MD; Manfred Cejna, MD; Larry Diaz-Sandoval, MD; Sean Willis, PhD; Laura Kirkwood, BSc; Peter W. Stratford, PhD; Anne B. Tietz, BS; Rudolf Kirchmair, MD; Marcy Silver, BS; Cindy Curry, BS; Andrea Wecker, BS; Young-Sup Yoon, MD; Regina Heidenreich, PhD; Allison Hanley, BS; Marianne Kearney, BS; Fermin O. Tio, MD; Patrik Kuenzler, MD; Jeffrey M. Isner, MD†; Douglas W. Losordo, MD

Background—Drug-eluting stents represent a useful strategy for the prevention of restenosis using various antiproliferative drugs. These strategies share the liability of impairing endothelial recovery, thereby altering the natural biology of the vessel wall and increasing the associated risk of stent thrombosis. Accordingly, we tested the hypothesis that local delivery via gene-eluting stent of naked plasmid DNA encoding for human vascular endothelial growth factor (VEGF)-2 could achieve similar reductions in neointima formation while accelerating, rather than inhibiting, reendothelialization.

Methods and Results—phVEGF 2-plasmid (100 or 200 μg per stent)—coated BiodivYsio phosphorylcholine polymer stents versus uncoated stents were deployed in a randomized, blinded fashion in iliac arteries of 40 normocholesterolemic and 16 hypercholesterolemic rabbits. Reendothelialization was nearly complete in the VEGF stent group after 10 days and was significantly greater than in control stents (98.7±1% versus 79.0±6%, P<0.01). At 3 months, intravascular ultrasound analysis revealed that lumen cross-sectional area (4.2±0.4 versus 2.27±0.3 mm², P<0.001) was significantly greater and percent cross-sectional narrowing was significantly lower (23.4±6 versus 51.2±10, P<0.001) in VEGF stents compared with control stents implanted in hypercholesterolemic rabbits. Transgene expression was detectable in the vessel wall along with improved functional recovery of stented segments, resulting in a 2.4-fold increase in NO production.

Conclusions—Acceleration of reendothelialization via VEGF-2 gene–eluting stents provides an alternative treatment strategy for the prevention of restenosis. VEGF-2 gene–eluting stents may be considered as a stand-alone or combination therapy. (Circulation. 2004;110:36-45.)

Key Words: gene therapy ■ endothelium ■ restenosis

Local drug delivery by drug-eluting stents may represent a useful strategy for the prevention of restenosis based on studies demonstrating promising results in animal models as well as clinical trials using antiproliferative drugs like rapamycin or taxol.1,2 However, these strategies share the liability of impairing endothelial recovery and increasing the associated risk of stent thrombosis.

Prior studies have suggested that acceleration of reendothelialization can attenuate restenosis and inhibit stent thrombosis.3–6 These effects have been mainly attributed to the potency of vascular endothelial growth factor (VEGF) to serve as an endothelial mitogen. However, delivery of naked plasmid DNA encoding for VEGF to the vessel wall using gene-eluting stents has not been demonstrated thus far.

Accordingly, we tested the hypothesis that delivery, via gene-eluting stent, of naked plasmid DNA encoding for human VEGF could achieve reductions in neointima formation while accelerating, rather than inhibiting, reendothelialization.

Methods

Animals
All procedures were performed under general anesthesia and sterile conditions in accordance with the St Elizabeth’s Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. New Zealand White rabbits weighing 4.5 to 5 kg with iliac artery dimension of 2.2±0.04 mm were used. A subset of animals (n=16) was placed on 1% cholesterol diet

Received November 1, 2002; de novo received February 23, 2004; revision received March 16, 2004; accepted March 19, 2004.

From the Department of Medicine (Cardiovascular Research), St Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Mass; Department of Pathology (F.O.T), University of Texas, San Antonio, Tex; Center for Learning and Memory (P.K.), Massachusetts Institute of Technology, Cambridge, Mass; and Biocompatibles UK Limited (S.W., L.K., P.W.S.), Surrey, UK.
†Deceased.
Correspondence to Douglas W. Losordo, MD, Chief of Cardiovascular Research, St Elizabeth’s Medical Center, 736 Cambridge St, Boston, MA 02135.
E-mail douglas.losordo@tufts.edu
© 2004 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000133324.38115.0A
with 3% peanut oil for 4 weeks before initial intervention, which was maintained throughout the follow-up period.

**BiodivYsio Phosphorylcholine Polymer Stents**
The 15-mm BiodivYsio stent was electropolished, cleaned, and coated with a phosphorylcholine polymer (PC)\(^7,8\) with or without phVEGF-2 plasmid (100 to 200 \(\mu\)g) under sterile conditions. Coating and manufacturing was performed by Biocompatibles UK Ltd, and the stent was premounted on a 3-mm balloon catheter covered by a 5F protection sleeve. The stents were shipped at room temperature and used within 6 months of manufacture. The stability and integrity of the plasmid were verified by sequencing of DNA eluted from randomly selected stents. No stability data are available on stents beyond 6 months because all stents were used within this time frame.

**phVEGF-2 Plasmid**
The plasmid phVEGF-2 (pVGII, Corautus Genetics) is a 5283-bp plasmid that contains the human VEGF-2 coding sequence.\(^9,10\)

**In Vivo Catheter Procedures**
After surgical exposure of the external carotid artery, a 5F introducer sheath (Radifocus, Terumo) was advanced to the lower abdominal aorta followed by administration of 1000 U heparin. Balloon denudation of the external iliac artery was performed by sequential withdrawal (6 times) with a 2F Fogarty balloon catheter (Baxter Edwards). Stent implantation was performed by balloon inflation (20 seconds at 10 atm). In a subset of rabbits, stents were implanted bilaterally. A single dose of aspirin 50 mg (Aspisol, Bayer) was administered intravenously after procedure. For follow-up angiograms, the contralateral carotid artery was exposed surgically.

**Intravascular Ultrasound Imaging and Analysis**
Intravascular ultrasound (IVUS) imaging was performed at baseline and 3 months follow-up using 2.5F 40-MHz transducers (Atlantis SR Plus, Boston Scientific Scimed) with motorized pull-back speed of 0.5 mm/s. Measurements were made twice every 1 mm and included in-stent area, lumen area, and intimal hyperplasia cross-sectional areas (CSAs).\(^11,12\)

**Histological and Ultrastructural Analysis**
Specimens were embedded in methyl methacrylate and cut with a diamond blade followed by metachromatic staining. For 6 different sections, the extent of vessel injury quantified by the method of Schwartz et al,\(^13\) inflammation score, and areas of neointima, media, native vessel lumen, and stent lumen were measured. Macrophages were detected by RAM-11 staining.

**Evaluation of Stent Endothelialization**

**Silver Staining and Scanning Electron Microscopy**
Reendothelialization was determined by silver staining and scanning electron microscopy as previously described.\(^5\)

**Evans Blue**
The extent of reendothelialization was observed by Evans blue staining\(^5\) in 2 animals. Seven microliters of 0.5% Evans blue was infused via the ear vein 30 minutes before euthanasia. After fixation in 100% methanol, the stent was opened and photographed.

**Rabbit Endothelial Progenitor Cell Assay**
Peripheral blood mononuclear cells were isolated from blood of rabbits at predetermined time points by density gradient centrifugation as described previously.\(^14\)

![Figure 1. IVUS analysis of VEGF-2 gene-eluting stents. Representative IVUS imaging at 3-month follow-up in VEGF stent (A) or control stent (B) implanted in hypercholesterolemic rabbit. C, Stent lumen CSA after stent implantation and lumen CSA as well as intimal hyperplasia for both groups at 3-month of follow-up. \(^*\)P<0.001.]
Detection of Transgene Expression

**RT-PCR**
RNA of whole-vessel segments was extracted using the RNAeasy Kit (Quiagen). cDNA synthesis was performed with 1 µg of total RNA using the Superscript II kit (Life Technologies) and Advantage-GC cDNA polymerase (Clontech). For semiquantification, QuantumRNA 18S internal standards were used (Ambion). Reverse transcription-polymerase chain reaction (RT-PCR) products were analyzed by 1% agarose gel electrophoresis.

Specific primers for human VEGF-2 were as follows: sense, 5’ACGAGCTCCTCAGCAAGACGTAT3’; antisense, 5’GGAGATCCATCTGTTGAGTCATCTCC3’. A 298-bp PCR product was identified, which was not detectable in extracts of rabbit iliac arteries from the control group or cultured rabbit smooth muscle cells (data not shown).

**In Situ Hybridization**
VEGF-2 RNA expression was localized by in situ hybridization of frozen tissue sections under RNAse-free conditions. Sense or antisense riboprobe were designed based on the above-mentioned primers using T7 RNA polymerase (Promega) and digoxigenin labeling (Roche). Hybridization of the riboprobes (20 ng/mL) was performed at 55°C for 18 hours. Vessel cross sections were incubated with sheep anti-DIG POD antibody (Roche) in TMB (100 mmol/L Tris HCl [pH 7.5], 150 mmol/L NaCl, 0.5% blocking reagent) 1:100 overnight at 4°C. Vessel cross sections were hybridized with sheep antiserum or amplification reagent (TSA, Plus Cy3 System, Perkin Elmer). Slides were mounted with Fluromount G (Southern Biotech Associates), and red fluorescent reaction products were visualized under fluorescent microscopy.

**VEGF Protein Expression in Tissue**

**Western Blotting**
Tissue samples from rabbit iliac arteries were homogenized in radioimmunoprecipitation assay lysis buffer and analyzed for VEGF-2 expression as described.10

**Measurement of NO From Vessel Segments**
NO production of excised stent segments was measured in an aerated organ bath containing Krebs buffer according to the modified Griess reaction (Sigma) as previously described.15

**Statistical Analysis**
All data are given as mean±SD or SEM as indicated. Continuous variables were compared by means of Student’s t test or Mann-Whitney U Test. Multiple comparisons were performed by Kruskal-Wallis test or ANOVA with Bonferroni’s correction using SPSS 9.0. A P value of <0.05 was considered significant.

**Results**

**Animal Characteristics**
A total of 62 stents were implanted in a randomized, blinded fashion in 56 male New Zealand White rabbits (n=16 hypercholesterolemic, mean cholesterol level 1349±220 mg/dL). Before stent implantation, denudation of the endothelium was performed. In 2 animals with acute follow-up, we demonstrated that balloon denudation with rotate/withdrawal technique was complete (98% to 100%) and reliable. Lumen diameter after stent implantation was 2.55±0.05, resulting in a stent/artery ratio of 1.16±0.04. Twenty-six BiodivYsio PC stents served as control group and were compared with PC stents coated with 100 µg VEGF-2 plasmid (n=18) or 200 µg (n=18).

Because no significant differences were observed between stents loaded with 100 or 200 µg plasmid DNA, VEGF-coated stents (n=36) were combined for additional analysis. There was no stent thrombosis in the follow-up period for either group.

**Quantitative IVUS Analysis**
Baseline and 3-month follow-up data of IVUS measurements were demonstrated in Figure 1. Postintervention measurements of stent and CSAs were similar in both groups (for normocholesterolemic rabbits, 6.2±0.4 versus 6.3±0.5 mm², P=0.7, n=10; for hypercholesterolemic rabbits, 5.7±0.4 versus 5.75±0.4 mm², P=0.7, n=10).

IVUS imaging demonstrated trends toward reduced intimal hyperplasia (1.21±0.5 versus 1.41±0.4 mm², P=0.5) and increased lumen areas (5.51±0.22 versus 4.88±0.68 mm², P=0.1) in VEGF stents implanted in normocholesterolemic animals (n=5 per group). In hypercholesterolemic rabbits, minimal lumen cross-sectional areas were significantly greater (4.2±0.4 versus 2.27±0.3 mm², P<0.001) and percent cross-sectional narrowing was significantly lower...
(23.4±6% versus 51.2±10%, \( P<0.001 \)) in VEGF stents (n=4) compared with control stents (n=4), as illustrated in Figure 1.

**Histomorphometric Analysis**

Morphometric analysis confirmed data obtained by IVUS, demonstrating a trend toward larger lumen cross-sectional areas and reduced neointimal hyperplasia (1.31±0.12 versus 1.42±0.19 mm², \( P=0.2 \)) in normocholesterolemic animals in the VEGF stent group (n=4) versus the control stent (n=4). However, in hypercholesterolemic animals (n=4), neointimal lesion cross-sectional area and percent area stenosis were significantly greater in the control stents (Figure 2) whereas lumen cross-sectional area was significantly greater in the VEGF-stented vessels at 3-month follow-up.

The extent of vessel injury at the stent site (injury score) was similar in both groups (for normocholesterolemic rabbits, 1.67±0.47 versus 1.61±0.5; for hypercholesterolemic rabbits, 2.1±0.3 versus 2.0±0; \( P=0.4 \)). There was no excess inflammation observed in the normocholesterolemic rabbits. In contrast, the inflammation score in the hypercholesterolemic animals receiving a VEGF-2–coated stent was slightly lower compared with uncoated PC stents (0.75±0.43 versus 1.33±1.1, \( P=0.1 \)).

**Reendothelialization**

Reendothelialization as assessed by Silver staining was nearly complete in the normocholesterolemic VEGF stent group after 10 days and was significantly greater than in control stents (98.7±1% versus 79±6%, \( P<0.01 \), n=4 and n=3 stents per group) (Figure 3). Reendothelialization was delayed in hypercholesterolemic animals receiving control stents; however, VEGF gene transfer to the vessel wall significantly accelerated the reendothelialization process at 10 days (81.87±4 versus 29.7±3%, \( P<0.01 \), n=12 segments of each of 2 stents per group), as illustrated by scanning electron microscopy (Figure 3).

Evans blue staining also supported that functional endothelialization appeared accelerated in the VEGF stent group (Figure 4).

**Functional Recovery of Endothelium**

(NO Measurements)

NO production by VEGF-2–treated arteries (n=3) was significantly greater than in control segments at 10 days after implantation, resulting in a 2.4-fold increase in NO production compared with vessel segments (n=3) with control stent implantation (0.122±0.018 versus 0.05±0.003 \( \mu \)mol/L, \( P<0.001 \)). These data reveal that the anatomic improvement in endothelial recovery was accompanied by enhanced local functional endothelial recovery induced by VEGF-2 gene transfer (Figure 4).

**Endothelial Progenitor Cell Assay**

Prior studies have revealed that recruitment of circulating endothelial progenitors is an important mechanism for endo-
to determine if VEGF gene transfer into the vessel wall via drug-eluting stent increases the number or differentiation of circulating endothelial progenitor cells (EPCs) systemically, rabbit EPC culture assays were performed. These assays documented an increase in circulating EPC number in rabbits receiving VEGF-2 stents compared with control stents, peaking at day 7 after stent implantation (88±6 versus 64±3 per mm² at day 7, P<0.01) (Figure 4D). Detection of Transgene Expression

The pVGI.I plasmid was detected in the vessel wall after VEGF-2 stent placement 24 hours after implantation. None of the remote organs disclosed evidence for systemic plasmid or transgene expression (Figure 5).

VEGF-2 gene expression was demonstrated by RT-PCR in the VEGF-stented iliac arteries as early as 72 hours, persisting for 10 days, and was undetectable after 2 weeks. (Figure 5). Detection of protein expression was performed by Western blotting for VEGF-2 from whole-vessel extracts (Figure 6).

Histological demonstration of human VEGF-2 RNA is provided by in situ hybridizations, indicating that mRNA expression was localized predominantly to the adventitia and outer media of the vascular wall (Figure 7). Immunofluorescent staining documented that most gene expression is by cells that are negative for CD31 expression, ie, the transfected cells are primarily not endothelial cells (Figure 8).

Discussion

Our results demonstrate the possibility of therapeutic local gene transfer using a stent platform technology and that VEGF-2 gene-eluting stents may be effective in the prevention of restenosis. The data show that VEGF-2 plasmid was effectively delivered to the vessel wall, where transcript and protein were detected for up to 10 days after stent implantation. Local VEGF-2 gene, delivered by PC-coated stents, not only improved early reendothelialization at the stented site but also reduced neointimal proliferation as assessed by IVUS at 3 months of follow-up. IVUS and histomorphometric assessment revealed significant differences in neointimal formation in hypercholesterolemic animals after implantation of VEGF stents compared with PC control stents. Moreover, and in contrast to all other currently marketed drug-eluting stents, VEGF-2–eluting stents result in acceleration of anatomic and functional endothelial recovery. These data, therefore, represent the first demonstration of an antirestenosis strategy using a gene-eluting stent, which ameliorates, rather than disrupts, the biology of the vessel wall after intervention.
Recent strategies to reduce restenosis using drug-coated stents have focused primarily on antiproliferative approaches. Although excellent results in animal models and clinical trials have been demonstrated, these strategies share the liability of impairing endothelial recovery and increasing the associated risk of stent thrombosis.17,18

Both endovascular radiation18 and drug-eluting antiproliferative stents show a decrease in neointimal growth in animal experiments. However, this is accompanied by delayed healing characterized by persistence of neointimal fibrin (with or without inflammation), a decrease in smooth muscle cells, and incomplete endothelialization.19

These findings indicate that the regenerative capacity of the vessel wall endothelial cells seems to be essential for the healing process and might be impaired in treatment strategies with antiproliferative agents.20,21

Prior studies have suggested that acceleration of reendothelialization by VEGF can consequently attenuate restenosis and inhibit stent thrombosis.3,22 Our findings are consistent with these prior data and additionally capitalize on the advent of technology that makes gene delivery a straightforward extension of the standard revascularization procedure.

The ability of locally delivered VEGF to accelerate reendothelialization has been primarily attributed to the ability of VEGF to serve as an endothelial mitogen. Similar effects on reendothelialization associated with suppression of neointimal...
mal proliferation have also been demonstrated for estradiol, tumor necrosis factor–soluble receptor, and HMG CoA reductase inhibitors, emphasizing the concept that acceleration of endothelial recovery may be an alternative strategy for the prevention of neointimal proliferation.

Indeed, we hereby provide insights into a potential mechanism accounting for the demonstrated effects seen with VEGF gene transfer delivered by gene-eluting stents. Improved endothelial recovery at the stented site after local VEGF gene transfer was demonstrated by Evans blue staining, silver staining, and scanning electron microscopy. These data are additionally supported by enhancement of functional recovery with an increase in NO production of VEGF-stented vessel segments after effective gene transfer to the injured vessel wall. The above findings were yielded from studies in normocholesterolemic rabbits. To investigate the potential for restenosis prevention, we used hypercholesterolemic rabbits generated by 1% cholesterol feeding. Although the cholesterol levels in these animals are far higher than those found clinically, this model has been shown to be more stringent, developing more aggressive neointimal lesions after angioplasty and stenting compared with the normocholesterolemic rabbit, and therefore more useful as a preclinical model.

VEGF gene transfer was more effective in reducing neointimal proliferation in hypercholesterolemic animals, a setting not only where control stents result in pronounced neointima formation but also where endothelial cell function is known to be impaired, thus providing a rationale for rescue therapy.

Several attempts in the past have been made to achieve local gene delivery via coated balloon catheters or infusion catheters using both naked plasmid DNA and adenoviral vectors for transfer of VEGF-1 or -2. Gene delivery from a DNA-releasing stent is a convenient approach that combines revascularization with gene delivery in a single procedure. The present study extends the findings of these previous investigations, now demonstrating for the first time that local gene delivery of a therapeutic gene via coated stents appears feasible, safe, and effective. VEGF-2 plasmid was only detected locally at the stented site within 24 hours of stent delivery. In addition, transgene expression was detectable locally up to 10 days after stent implantation, indicating that temporal transgene expression within a few days is sufficient to augment reendothelialization and thereby block subsequent neointimal proliferation. Histological evidence for human VEGF-2 RNA is provided by in situ hybridization, indicating that mRNA expression was localized predominantly to the adventitia and outer media of the vascular wall.

Neointimal thickening has been inferred to result most often from vascular smooth muscle cell (VSMC) proliferation. As a result, intense effort has been applied to discern the mechanisms that govern VSMC proliferation after angioplasty and to develop therapies to inhibit VSMC growth. The enthusiasm for this field is reflected by the 857 manuscripts (to date) that have been published examining the role of SMC proliferation in restenosis and, most importantly, by the advent of approaches that have demonstrated significant degrees of clinical success in addressing this aspect of the response to injury. Both brachytherapy and drug-eluting stents target proliferating VSMCs at the site of injury and have been successful in reducing neointimal lesion formation. However, the fate of the endothelium in humans after radiation or drug-eluting stent is uncertain at present, although evidence exists to suggest that endothelial recovery may be perturbed. The initial applications of intravascular brachytherapy were complicated by a significant incidence of

Figure 7. Transgene expression (in situ hybridization). Localization of VEGF-2 was detected by hybridization probes on longitudinal rabbit iliac artery sections (stent was carefully removed and sections were frozen). Note expression of VEGF-2 mRNA (red fluorescence) in adventitia and outer media. Similar results were obtained at days 5 and 10.
The present study is limited to VEGF gene delivery in doses of 100 to 200 μg, a very narrow dose range in which no differences were detected between the doses. Additional studies are necessary to demonstrate dose dependency as well as influence of various polymers on plasmid release and kinetics. Another important limitation lies in the fact that the data regarding gene expression and EPC mobilization were derived from studies in normcholesterolemic rabbits, whereas the studies examining neointimal proliferation were in both subsets emphasizing hypercholesterolemic rabbits.

In summary, the present study constitutes an alternative and novel treatment strategy, acceleration of reendothelialization via VEGF-2 gene-eluting stents, for the prevention of restenosis. The data also provide additional evidence for the role of circulating endothelial progenitors in the process of endothelial repair.
This strategy addresses the liability common to all current strategies of restenosis prevention and may be considered as a stand-alone or combination therapy. Additional preclinical or clinical investigations of VEGF-plasmid–coated stents for local gene delivery are warranted.

Acknowledgments
This study is dedicated to our inspiring mentor, the late Jeffrey M. Isner. This study was supported in part by NIH grants (HL-53534, HL-57515, HL-60911, HL-63414, HL-63695, and HL-66957) and the Shaugnessy center for clinical genetics. Dr. Walter is the recipient of a research grant from the Deutsche Forschungsgemeinschaft (WA 1461/1). Dr. Cejna is a recipient of a research grant from the Fonds der Chemischen Industrie. Dr. Khatri is a recipient of a research grant from the Fonds der Chemischen Industrie. We gratefully acknowledge the expert technical assistance of Hong Ma and Lindsay Heyd and the expert administrative assistance of Mickey Neely and Deirdre Costello.

References
Local Gene Transfer of phVEGF-2 Plasmid by Gene-Eluting Stents: An Alternative Strategy for Inhibition of Restenosis

Dirk H. Walter, Manfred Cejna, Larry Diaz-Sandoval, Sean Willis, Laura Kirkwood, Peter W. Stratford, Anne B. Tietz, Rudolf Kirchmair, Marcy Silver, Cindy Curry, Andrea Wecker, Young-Sup Yoon, Regina Heidenreich, Allison Hanley, Marianne Kearney, Fermin O. Tio, Patrik Kuenzler, Jeffrey M. Isner and Douglas W. Losordo

Circulation. 2004;110:36-45; originally published online June 21, 2004;
doi: 10.1161/01.CIR.0000133324.38115.0A
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/110/1/36

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/