Reduction of Vein Graft Disease Using Photodynamic Therapy with Motexafin Lutetium in a Rodent Isograft Model

Atsushi Yamaguchi, MD, PhD; Kathryn W. Woodburn, PhD; Motoya Hayase, MD; Robert C. Robbins, MD

Background—Motexafin lutetium (Lu-Tex) is a photosensitizer that targets atheromatous plaque. Subsequent photoactivation (photodynamic therapy [PDT]) induces local cytotoxic effects. The aim of the present study was to investigate whether Lu-Tex targets vein graft intimal hyperplasia and whether subsequent photoactivation attenuates the disease process.

Methods and Results—The subcellular localization of Lu-Tex and postillumination viability were studied in cultured human vein graft smooth muscle cells. Inferior vena cava–grafted rats were injected with Lu-Tex (10 mg/kg) 4 or 12 weeks after grafting. Biodistribution was assessed in a subgroup 24 hours after administration. Light therapy (742 nm) was performed 24 hours after Lu-Tex injection by illuminating intraperitoneally placed isografts using a laparotomy. Animals were divided into the following 4 groups: PDT (n = 15), Lu-Tex injection and laparotomy (n = 13), light treatment (n = 14), and laparotomy only (n = 13). Grafts were harvested 14 days after treatment for histochemical analysis. Lu-Tex localized within subcellular organelles of smooth muscle cells, and subsequent photoactivation induced cell death via apoptosis. The Lu-Tex concentrations present in the vein grafts were 9.3 times higher than those in the normal inferior vena cava. Postoperative PDT at 4 weeks after surgery significantly reduced the intima/media ratio, whereas treatment at 12 weeks did not reduce the intima/media ratio. Activated macrophages were observed 4 weeks after grafting; however, a significant reduction occurred in these cells by 12 weeks. The mechanism by which PDT works may be related to the presence of activated macrophages.

Conclusions—PDT significantly reduces the intima/media ratio in the early phase of vein graft disease. Lu-Tex–mediated PDT may be a viable method for the attenuation of atherosclerotic disease in vein grafts. (Circulation. 2000;102[Suppl III]:III-275-III-280.)

Key Words: angioplasty ■ bypass ■ grafting ■ coronary disease ■ lasers

Intimal thickening of veins implanted into the arterial system of dogs was described by Carrel and Guthrie in 1906. Today, accelerated graft atherosclerosis of the saphenous vein remains troublesome for patients after coronary artery bypass surgery. The angiographic evaluation of vein grafts in patients after bypass surgery shows occlusion rates of 12%, 19%, 25%, 40%, and 50% at 1 month and at 1, 5, 10, and 15 years, respectively. Despite the high incidence of graft failure, autologous saphenous vein grafts remain the most widely used conduits for coronary artery bypass surgery. This is because of their ready availability and ease of removal and the insufficient number of arterial grafts available to achieve complete revascularization in patients with multivessel coronary disease. Although advances in surgical technique and interventional therapies, such as percutaneous transluminal angioplasty, directional atherectomy, stent placement, and excimer laser angioplasty, have all undoubtedly had a salutary impact on vein graft patency, novel approaches for the prevention of accelerated vein graft intimal hyperplasia need to be explored.

Photodynamic therapy (PDT) is a therapeutic modality that uses nonthermal light to activate photosensitizers that have accumulated in diseased tissue. Free radical species are produced either by the photosensitizer itself or by energy transfer to molecular oxygen to produce singlet oxygen; both processes result in cytotoxic effects on cellular and tissue structures. Motexafin lutetium (Lu-Tex) is a second-generation photosensitizer that is activated by tissue- and blood-penetrating far red light (~730 nm), and it is presently in phase II clinical trials for recurrent breast cancer. In addition to its oncological indications, Lu-Tex has many vascular disease applications. Lu-Tex localized in the atherosclerotic plaque of hyperlipidemic rabbits and, after photoactivation, it was effective in reducing plaque burden. A clinical trial evaluating patients with peripheral obstructive disease is ongoing, and it uses a procedure now known as photoangioplasty.
We demonstrated the subcellular localization of Lu-Tex within human coronary vein graft smooth muscle cells (SMCs) using fluorescence microscopy and assessed cell survival after photoactivation. In addition, we assessed the tissue biodistribution of Lu-Tex after administration in a rodent isograft model. The overall objective of the present study was to determine whether light therapy will have cytotoxic effects on the cellular systems within the neointima.

Methods

Chemicals
The synthesis and chemical characterization of motexafin lutetium (also known as lutetium texaphyrin; Lu-Tex) used in this study has been described previously. The drug was formulated in 5% aqueous mannitol (pH adjusted to 5.5) to yield a final concentration of 2.2 mg/mL. Fluorescent probes for staining the mitochondria (MitoTracker Green FM), lysosomes (LysoTracker red DND-99), endoplasmic reticulum (Rhodamine B, hexyl ester), and nucleus (Hoechst 33342, HO342) were purchased from Molecular Probes.

Cells
Human SMCs from a coronary vein bypass graft were obtained from a subject who had received the graft 1 year before dying from a myocardial infarction (Clonetics). Cells were grown in cell line–specific growth media according to the instructions supplied by Clonetics (SMGM-2 bullet kit).

Fluorescence Microscopy
The SMCs were seeded (4X10^3) on Laboratory-Tek 10 cm^2 Flaskette glass chamber slides (Nunc, Inc) containing 4 mL of media. The cells were allowed to incubate for 24 hours to enable attachment to the substratum. Lu-Tex was added to the media to yield a final concentration of 10 μg/mL and incubated for predetermined amounts of time (1 to 24 hours). After incubation, the cells were viewed with a 63X oil-immersion objective with a Zeiss Axiosplan-2 microscope. Filter cubes, matched to the excitation and emission spectra of each fluorophore, were used. The following dye concentrations and incubation times were used for staining specific organelles: 0.5 μg/mL HO342 for 10 minutes; 500 nmol/L Rhodamine B for 30 minutes; 50 nmol/L LysoTracker for 1 hour; and 250 nmol/L MitoTracker Green for 30 minutes. Phase contrast and fluorescence images were stored electronically and color-processed using the EASY FISH program (Applied Spectral Imaging).

In Vitro Viability Assays
Cells were seeded (2X10^5) into 96-well cell culture plates and allowed to incubate overnight to enable adherence. Lu-Tex was added to the wells (0 to 86 μg/mL) and incubated for 3 hours (n=6) or 24 hours (n=8). Then, the media was removed, cells were washed once with PBS, and fresh media was added. Cells were exposed to light using a light-emitting diode array (model QB1310CS-728 to 728, Quantum Devices; λmax, 729 nm; full-width half maximal wavelength, 33 nm) using a fluence of 2 J/cm^2 at a rate of 4.2 mW/cm^2. Plates were returned to the incubator, and cell viability studies were performed 3 days later using an MTT assay. Changes in nuclear morphology were studied using the nuclear probe HO342 (1 μg/mL for 10 minutes) in cells both before and after PDT treatment.

Vein Grafting
Adult male Lewis rats (8 to 10 weeks old; 230 to 270 g) were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Research Council. Both donor and recipient animals were anesthetized in an induction chamber with the inhalation agent methoxyflurane; this was followed by an intraperitoneal injection of pentobarbital (50 mg/kg). Heparin (400 U/kg) was injected into the donor animal via the retroperitoneal inferior vena cava (IVC), and the animal was exsanguinated by dividing the abdominal vessels. Then, a 1-cm segment of supradiaphragmatic IVC was excised using a median sternotomy.

The abdominal aorta of the recipient was isolated through a midline incision. The donor vein graft was anastomosed to the recipient abdominal aorta in an end-to-side fashion using 8-0 Prolene sutures. After both ends of the vein graft were anastomosed to the abdominal aorta, the aorta was ligated with an 8-0 Prolene suture between the 2 anastomoses. The patency of the vein grafts was confirmed by palpation of the femoral pulse and inspection of the legs.

Postoperative Treatments
Postoperative treatments were performed 4 or 12 weeks after bypass grafting. Animals were injected with 10 mg/kg Lu-Tex via the tail vein. Light illumination was delivered to the intra-abdominally placed isografts via a laparotomy 24 hours after drug administration. A light-emitting diode (QBEAM 2001, Quantum Devices Inc; λmax, 742 nm; full-width half maximal wavelength, 30 nm) was used to deliver light using a light fluence of 75 J/cm^2 at a rate of 75 mW/cm^2, which equates to 16 minutes and 40 seconds.

Animals were divided into the following 4 groups according to postoperative treatment: PDT with both Lu-Tex injection and light therapy (PDT, n=15), Lu-Tex injection and laparotomy without light therapy (drug only, n=13), laparotomy with light therapy only (light only, n=14), and laparotomy only (laparotomy, n=13).

Biodistribution
Four weeks after grafting, 3 animals were injected with 10 mg/kg Lu-Tex and killed 24 hours after administration to determine the Lu-Tex content of plasma and specific organs. One additional animal was injected with 5% mannitol to serve as a vehicle-alone control. The Lu-Tex concentration was determined by fluorescence spectroscopy using a previously published method.

Morphometric Analysis
Animals were killed 14 days after the postoperative treatments. After exsanguination, the vein grafts were pressurized at 90 mm Hg with a 10% buffered formalin solution for 15 minutes. The grafts were then immersed in 10% buffered formalin for 15 minutes. The vein grafts were processed using established histological techniques that are based on an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Peroxidase was visualized with diaminobenzidine horseradish peroxidase complex (DAB substrate kit, Vector Laboratories). The capillaries were used to measure the intimal and medial areas of each graft. The intima/media ratio was calculated by dividing the intimal area by the medial area.

Immunohistological Analysis
Immunohistochemical staining with anti-rat macrophage antibody (ED1; Serotec) and anti-α-SMC actin monoclonal antibody (Sigma) was performed in the paraffin sections (5 μm) from the vein grafts using an established technique that is based on an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Peroxidase was visualized with diaminobenzidine tetrahydrochloride (DAB substrate kit, Vector Laboratories).

Differences in the distribution of immunopositive cells were estimated by a computerized image analysis system after defining the neo-intimal area for each vessel using sequential elastin-stained sections, the ED1 and α-SMC actin immunopositive areas were detected by measuring the area encompassed by pixels of the color intensity of the immunopositive cells.

Statistical Analysis
Data are expressed as mean±SD. The comparisons between >2 groups were made using multivariate ANOVA of independent

Animals
Adult Lewis male rats (8 to 10 weeks old; 230 to 270 g) were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Research Council.
groups to determine the overall difference, followed by a post hoc Bonferroni/Dunn test to determine statistical significance between groups. The comparisons between 2 groups or 2 time points were made by an unpaired t test. Statistical significance was accepted at the 95% significance level (P<0.05).

Results

Subcellular Localization and Phototoxicity
Lu-Tex partitioned into the subcellular organelles of human vein graft SMCs. The subcellular localization of 10 μg/mL Lu-Tex is displayed in Figure 1b after 24 hours of incubation. Costaining with organelle markers identified the subcellular organelles as mainly lysosomes and endoplasmic reticulum, with some partitioning within the mitochondria. Lu-Tex alone (up to 86 μmol/L) did not cause any cytotoxicity in the adherent SMCs after incubation for either 3 or 24 hours, as assessed using an MTT assay 72 hours after treatment. The dependence of phototoxicity on Lu-Tex concentration is shown in Figure 2. PDT did induce cell death; lethality was marginally more pronounced after 24 hours of incubation compared with 3 hours (data not shown; LD_{50} 2 and 1 μmol/L at 3 and 24 hours, respectively). Nuclear morphology was probed using HO342 staining. After PDT, the classic hallmark

Figure 1. Subcellular localization of Lu-Tex (10 μg/mL) within human coronary vein graft SMCs after 24 hours. The transmission micrograph is shown in a, the Lu-Tex localization pattern (red) is displayed in b, and a multiple-exposure image with organelle probes specific for the nuclei (blue), mitochondria (green), and Lu-Tex (red) is shown in c. Lu-Tex partitions within the lysosomes, endoplasmic reticulum, and mitochondria. Scale bar is 10 μm.

Figure 2. Loss of cell viability after PDT in human vein graft SMCs (left). Cells were incubated with varying concentrations of Lu-Tex for 24 hours and then exposed to 2 J/cm² of 729-nm light at a rate of 4.2 mW/cm². Cells were stained with a nuclear morphological marker dye (HO342) before (top right) and after PDT (bottom right). The hallmark morphological features of apoptosis were observed after PDT (bottom right). Data are reported as mean±SD for 8 samples. Scale bar is 10 μm.
morphological features of apoptosis (chromatin condensation and nuclear blebbing) were observed (Figure 2c).

Drug Biodistribution
The distribution of Lu-Tex within specific tissues and the plasma of vein-grafted rats is shown in Figure 3. A single animal served as a vehicle-alone control, and no fluorescence signal (700 to 800 nm) was observed in any of these samples, thus confirming the validity of the fluorescence method. Lu-Tex was concentrated in the atherosclerotic plaque in the vein grafts (Figure 3), and it was present in the graft in concentrations 9-fold of those in the IVC. Little sensitizer was observed in heart and muscle tissue, and none was detected in plasma. High levels were detected in the liver, kidney, and spleen.

Morphometric Analysis of Intimal Hyperplasia
Figure 4 shows the cross sections of the vein grafts stained with elastin. The Table lists the data from the morphometric analysis of the vein grafts. In the PDT group, the intimal area in the vein grafts treated at 12 weeks after surgery was significantly greater than that at 4 weeks after surgery (P<0.05), whereas the medial area significantly decreased during these 2 time points (P<0.01), which resulted in a significant increase of the intima/media ratio with time (P<0.01). In the other study groups, no significant changes in the intima/media ratio were observed, although mean values of the medial area significantly decreased between the time points.

When the postoperative treatments were performed 4 weeks after vein grafting, the mean intima/media ratio in the PDT group was significantly lower than the corresponding values in the other study groups (P<0.05); however, the mean intima/media ratios in the vein grafts treated at 12 weeks after surgery had no significant differences between the study groups.

ED1 and α-SMC Actin Contributions to the Neointima
The expanded neointima containing ED1- and α-SMC actin–positive cells is displayed in Figure 5. IVC-grafted rats that were treated with PDT 4 weeks after grafting exhibited a decrease in macrophages (Figure 5a), as defined by ED1-positive cells, compared with those rats that experienced a laparotomy procedure alone (Figure 5b). The SMCs also decreased in the PDT group (Figure 5c) compared with the laparotomy control group (Figure 5 days). In each study group, quantitative analysis of the ED1 immunopositive area showed a significant decrease in the contribution of macrophages within the neointima in the vein grafts treated at 12 weeks after surgery compared with those at 4 weeks after surgery (P<0.01).

When the postoperative treatments were performed at 4 weeks after surgery, PDT reduced the ED1 immunopositive area in the neointima; PDT treatment was nearly statistically significantly different (P=0.07) from the other study groups. PDT performed at 4 weeks after surgery significantly decreased the contribution of α-SMC actin within the neointima in the vein grafts compared with the other study groups (P<0.05). However, PDT failed to reduce either ED1 or α-SMC actin immunopositive area in the neointima of the vein grafts when the treatment was performed at 12 weeks after vein grafting.

Discussion
Accelerated atherosclerosis remains the major complication after coronary artery bypass graft surgery. Vein graft failure incorporates 3 major, time-dependent mechanisms. Thrombosis appears within 1 month of operation, intimal prolifer-
ative hyperplasia becomes prominent beyond 1 month, and late failure follows as a result of progressive atherosclerosis. Analyses of saphenous vein grafts from patients several years after coronary artery bypass grafting have documented accelerated intimal hyperplasia.\textsuperscript{14,15} Vein graft segments immediately after harvest (at the time of operation) show focal endothelial denudation throughout the length of the harvested vein segments. Surgical handling and exposure to the shear force of the arterial circulation causes injury to the endothelium and initiates a rapid infiltration of inflammatory cells into the subendothelial tissues and media, causing a fibrous coating to form over the graft surface. Within 4 days of implantation, macrophages can be detected in the intima and media.\textsuperscript{16} Fibroblasts and SMCs proliferate in the subendothelial portion of the intima within 1 month of implantation, causing intimal and medial thickening. Thereafter, the smooth muscle fibers of the media gradually diminish and are replaced, in part or whole, by fibrous tissue. Fibrous tissue increases in the adventitia, and the elastic fibers are disrupted or disappear completely. Overall, the vein graft used as a pseudoartery becomes a “stiff, fibrous-tissue conduit” within 2 months of implantation.\textsuperscript{14,15}

In this study, Lu-Tex–mediated PDT was effective in reducing the intima/media ratio when the treatment was performed 4 weeks after vein grafting, and a reduction in the \(\alpha\)-SMC actin–positive area was also observed. The treatment was, however, not effective when applied 12 weeks after vein graft surgery. Planimetry analysis revealed that the medial area had attenuated at 12 weeks after grafting. It is highly plausible that the PDT cellular targets were no longer present in the lesions and, instead, the lesion was comprised of fibrous, PDT-inert material.

PDT efficacy is related to the precise localization of the sensitizer within diseased tissue because, after photoactivation, cytotoxic singlet oxygen is produced. Singlet oxygen has a minute reactive biological path length (\(\text{\(0.2\)}\) mm), so the discernment of the subcellular localization of a specific sensitizer is crucial in elucidating the mechanism of action.\textsuperscript{6} In this study, fluorescence microscopy revealed Lu-Tex uptake in lysosomes, the endoplasmic reticulum, and some mitochondria of human coronary bypass SMCs. Lu-Tex–mediated PDT was quite effective in killing SMCs via an apoptotic mechanism. This finding agrees with a previous study\textsuperscript{17} in which murine

### Morphometric Analysis of Vein Grafts

<table>
<thead>
<tr>
<th>Intimal Area, (\text{mm}^2)</th>
<th>Medial Area, (\text{mm}^2)</th>
<th>Intima/Media Ratio</th>
<th>ED1-Positive Area, (\mu\text{m}^2)</th>
<th>(\alpha)-SMC Actin-Positive Area, (\mu\text{m}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDT (n=9)</td>
<td>0.074±0.041</td>
<td>0.221±0.071</td>
<td>0.36±0.23*</td>
<td>2422±1866</td>
</tr>
<tr>
<td>Drug only (n=7)</td>
<td>0.103±0.051</td>
<td>0.181±0.060</td>
<td>0.68±0.035</td>
<td>3048±2031</td>
</tr>
<tr>
<td>Light only (n=7)</td>
<td>0.094±0.082</td>
<td>0.206±0.071</td>
<td>0.54±0.34</td>
<td>2701±2795</td>
</tr>
<tr>
<td>Laparotomy (n=7)</td>
<td>0.108±0.070</td>
<td>0.222±0.111</td>
<td>0.61±0.44</td>
<td>3882±2466</td>
</tr>
<tr>
<td>12 Weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDT (n=6)</td>
<td>0.114±0.071†</td>
<td>0.166±0.050\‡</td>
<td>0.77±0.53\‡</td>
<td>629±456\†</td>
</tr>
<tr>
<td>Drug only (n=6)</td>
<td>0.134±0.108</td>
<td>0.160±0.054\†</td>
<td>0.83±0.47</td>
<td>1480±908\‡</td>
</tr>
<tr>
<td>Light only (n=7)</td>
<td>0.102±0.059</td>
<td>0.146±0.065\‡</td>
<td>0.99±1.43</td>
<td>983±348\‡</td>
</tr>
<tr>
<td>Laparotomy (n=6)</td>
<td>0.108±0.047</td>
<td>0.139±0.056\‡</td>
<td>0.88±0.62</td>
<td>668±195\‡</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*\(P<0.05\) vs all other study groups (ANOVA); †\(P<0.05\) between 4 and 12 weeks in each study group; ‡\(P<0.01\) between 4 and 12 weeks in each study group.

Figure 5. The expanded neointima contained ED1 and \(\alpha\)-SMC actin–positive cells. Arrows indicate border between neointima and media. The vein graft treated with PDT has a minimal contribution from ED1-positive cells (a) and \(\alpha\)-SMC actin–positive cells (c). ED1-positive cells localize between the intima and media in vein graft treated with laparotomy only (b). \(\alpha\)-SMC actin–positive cells are observed in entire area of vein graft treated with laparotomy only (d). Original magnification, ×100.
leukemia L1210 and hepatoma cells that were photoactivated after Lu-Tex incubation caused an apoptotic response after lysosomal photodamage. In the study, the lysosomal enzyme cathepsin B was released into the cytoplasm; this caused a loss in the mitochondrial membrane potential, a release of cytochrome C (which elicited caspase-3 activation) and, finally, an apoptotic response.17

Lu-Tex has an enhanced affinity for atherosclerotic plaque in cholesterol-fed rabbits; subsequent light activation is effective in reducing plaque burden.9 PDT with 2 other photosensitizers, a phthalocyanine18 and methylene blue,19 is also effective in reducing experimental intimal hyperplasia in vein grafts when the therapy is applied after harvest and immediately before grafting. In the present study, Lu-Tex–mediated PDT was applied to treat vein grafts in which intimal hyperplasia was established. The concentration of Lu-Tex in the vein graft was 9.3 times higher at 24 hours after the injection than concentrations in the normal IVC, and it was 2 or 3 times higher than concentrations in the heart, lung, muscle, or skin. Lu-Tex exhibits a high affinity for plasma lipoproteins.9 Therefore, organs that are part of the reticuloendothelial system (liver, kidney, and spleen) and are involved in serum protein function and transport also take up Lu-Tex. However, retention of Lu-Tex by these organs at these concentrations is not likely toxic unless it is activated by light.

Neointimal thickening after vein grafting is mediated by mononuclear cell infiltration and activation.20 Macrophages present early in the reparative process and express growth factors that stimulate endothelial growth, especially under a hypoxic milieu or endothelial dysfunction.21 Two previous studies, Lu-Tex–mediated PDT was effective in reducing macrophages in a balloon injury model of atherosclerosis.23 In the present study, immunohistochemical staining with ED1 demonstrated upregulation of macrophages in the neointima at 4 weeks after surgery, with numbers diminishing at 12 weeks. The upregulation and downregulation of macrophages may be associated with the remarkable effect of PDT at 4 weeks, with no significant PDT effect observed at 12 weeks after vein grafting. The ED1-positive area in the neointima was decreased by PDT treatment at 4 weeks after surgery, and this decrease was on the verge of statistical significance (P=0.07) compared with the other study groups. The latter implies that macrophages are a critical PDT target, the depletion of which may cause vascular disease cessation and stabilization.

Two previous studies showed intimal hyperplasia suppression when therapy was given before surgical placement of the vein graft into the recipient.18,19 Because macrophages play a crucial role in atherogenic progression, it is feasible that this is the critical target and that prevention of monocyte recruitment into the lesion will arrest disease.25 Future studies will address the validity of this proposal and whether PDT downregulates the expression of cellular adhesion and chemoattractant proteins, thereby suppressing the recruitment of the inflammatory cells that are responsible for disease development. From this study, we concluded that Lu-Tex–mediated PDT significantly reduced the intima/media ratio in vein grafts when the treatment was given at an early phase of vein graft disease.

References

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