Photodynamic therapy of vein grafts: Suppression of intimal hyperplasia of the vein graft but not the anastomosis

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Purpose: There is no clinically useful therapy for the suppression of vein bypass graft intimal hyperplasia (IH). Photodynamic therapy (PDT), a technique that uses light to activate otherwise biologically inert photosensitizers to produce cytotoxic effects, has been demonstrated to successfully inhibit experimental IH in balloon-injured arteries. The purpose of this study was to investigate the efficacy of PDT as a method to reduce vein graft IH.

Methods: Reversed external jugular vein bypass grafts of the common carotid artery were performed in 28 male Sprague-Dawley rats. The animals received either chloroaluminum sulfonated phthalocyanine (2.5 mg/kg intravenously) 24 hours before the ex vivo irradiation of the vein grafts (VG) with 100 joule/cm^2 at 675 nm (PDT VG) or saline solution as control (CON VG). Preharvest bromodeoxyuridine was administered to label proliferating cells. All vein grafts were perfusion fixed within 96 hours for a pilot study or at 2 and 4 weeks for the main study. Histology, immunohistochemistry, and morphometric analysis were performed.

Results: There was no acute thrombus formation in the hypocellular PDT VG with occasional platelets but no leukocytes adherent to the luminal surface. Intimal areas of the PDT VG were 18% at 2 weeks and 53% at 4 weeks of the CON VGs ($p < 0.05$). Medial areas and percent of stenoses were also significantly less in PDT than in CON VG. However, intimal hyperplasia noted in the longitudinal sections within 2 mm of the anastomoses did not demonstrate a difference between PDT and CON VG. Intimal hyperplasia of both PDT and CON VG consisted of smooth muscle cells, verified by immunohistochemistry. Bromodeoxyuridine-labeled cells were more abundant in 2-week than in 4-week specimens, were found most frequently in the intimal areas of the CON VG body, and were equivalent in the anastomoses of PDT VG and CON VG.

Conclusions: These data suggest that PDT of vein grafts suppresses the development of IH in the body of the vein graft but does not affect IH adjacent to the anastomoses. The artery may be the source of proliferating smooth muscle cells that contribute to the anastomotic vein graft IH. (J VASC SURG 1995;21:882-90.)

The saphenous vein is accepted as the best conduit for small- and medium-sized arterial reconstruction.[1] Nevertheless, even this conduit is not ideal. In fact, vein grafts used for femoropopliteal reconstruction have been reported to develop an occlusion rate of 30% to 35% during the first 2 to 3 years after implantation.[2] Studies of aortocoronary vein grafts found occlusion rates of 4% to 12% at 2 weeks and at 2 months, with a yearly attrition rate of 2% to 5%.[3,4] The main causes of the vein graft failure were stenotic lesions in the body of the graft and at the anastomoses. There was no reported correlation of vein graft failure to valve sites, tributaries, clamp sites, or residual valve cusps.[5] Stenotic lesions resulted most frequently from intimal hyperplasia (IH) and were responsible for 65% to 75% of all vein graft failures that occurred within the first postoperative year.[6]

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Table I. Morphometric analysis of VG

	*2 weeks**		*4 weeks**	
	CON VG (n = 4)	*PDT VG (n = 5)*	*CON VG (n = 5)*	*PDT VG (n = 4)*
Intimal area (mm^2)	0.148 ± 0.109	0.029 ± 0.011†	0.124 ± 0.038	0.066 ± 0.027†‡
Medial area (mm^2)	0.174 ± 0.037	0.114 ± 0.034†	0.145 ± 0.024	0.085 ± 0.020†
Luminal stenosis (%)	6.8 ± 3.4	1.6 ± 0.7†	12.4 ± 3.4‡	5.3 ± 2.2†‡
Diameter (mm)	1.69 ± 0.16	1.55 ± 0.14	1.13 ± 0.07‡	1.32 ± 0.26
BrdU anastomosis§	9.33 ± 2.05	16.5 ± 3.41	7.67 ± 1.85	4.67 ± 1.72
BrdU vein graft body§	7.17 ± 2.04	1.33 ± 0.54	4.00 ± 0.47	1.17 ± 0.4

BrdU, Bromodeoxyruidine.
*Mean ± SEM.
†$p < 0.05$ CON VG versus PDT VG.
‡$p < 0.05$ 2 weeks versus 4 weeks.
§Cells per 400 × field.

IH, a response to various types of vascular wall injuries, is the narrowing of the vascular lumen resulting from smooth muscle cell (SMC) migration to the subendothelial space and proliferation. This multifactorial process is triggered and regulated by numerous growth factors and cytokines.[7] Subsequent production of matrix contributes to the development of a luminal stenosis.

In vein grafts, two types of IH are described. The first is IH due to migration and proliferation of the vein's own medial SMC in the body of the graft, and this entity is represented as truncal IH. The second entity is anastomotic IH caused by SMC at and across the anastomoses, which may be derived, in part, from the artery and the vein graft.[8-10] Causes of nonacute vein graft stenoses were found to be from anastomotic IH (62%), atherosclerotic changes (20%), and external compression of the graft (10%).[11]

Although the mechanisms of IH are yet to be fully understood, a number of drugs have been tested for the prevention of vein graft IH. Some of these drugs such as heparin[12-15] have demonstrated inconclusive results, whereas others such as angiotensin-converting enzyme inhibitors, fish oil, and angiopeptin have no proven clinical efficacy.[16-18]

Photodynamic therapy (PDT), another approach being investigated for the inhibition of experimental IH, is a biologic application of photochemistry.[19-23] Light energy is delivered to tissue where it activates previously administered, otherwise inert, photosensitive drugs. Once light activates the photosensitizer, free radical moieties are produced, either by the photosensitizer itself or by energy transfer to ambient O_2. These free radicals result in cytotoxic effects.

Many photosensitizer drugs are available for PDT photochemistry. Chloroaluminum sulfonated phthalocyanine (CASPc) is a powerful second generation photosensitizer that, at therapeutic systemic doses, has no skin photosensitivity, a problem noted with many porphyrin photosensitizers.[19]

PDT has been demonstrated to decrease cell proliferation in several diseases, including experimental and human cancer.[24-26] For the inhibition of experimental IH, PDT depletes SMC without apparent compromise to the arterial wall. Despite the elimination of SMC in the artery wall, there is no visible cellular inflammatory response and no arterial wall degeneration but cellular repopulation of the arterial scaffold over time.[23,27]

Strong evidence suggests that PDT can inhibit experimental IH in the different models of balloon-injured arteries. This study was designed to determine whether PDT might also have a role in the treatment of vein graft IH.

METHODS

Surgery. Twenty-eight male Sprague-Dawley rats weighing 350 to 400 gm (Charles River Laboratories, Wilmington, Mass.) were anaesthetized with intramuscular ketamine (75 mg/kg) and xylazine (5 mg/kg). With an operating microscope (Codman & Shurtlett, Inc., Randolph, Mass.) the distal segment of the left external jugular vein (Fig. 1) was carefully harvested, gently placed on a PE-10 tube stent, and immersed in Ringer's lactate solution containing heparin (10 U/ml). For PDT, the vein grafts of the experimental group (PDT VG) were irradiated, whereas the control veins (CON VG) remained in phosphate-buffered saline solution without irradiation for a comparable time (25 minutes). Subsequently, a portion of the left common carotid artery was resected before replacement with the reversed vein interposition graft. The anastomoses were performed with interrupted 10-0 nylon sutures (Ethicon, Inc., Somerville, N.J.). There was no size

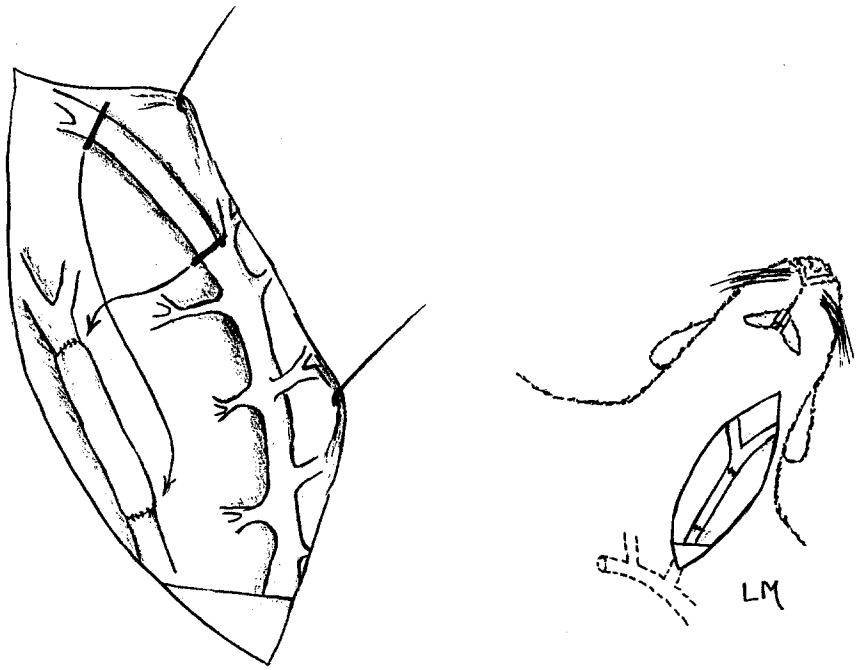

Fig. 1. Schematic of model used. Distal external jugular vein, as it proceeds deep into neck, is harvested, reversed, and sutured to cervical common carotid artery as bypass graft.

mismatch evident between the vein graft and artery after reconstituting blood flow.

PDT regimen. Twenty-four hours before carotid artery bypass surgery, animals of the PDT-treated group were injected intravenously with 2.5 mg/kg CASPc (CIBA-GEIGY, Basel, Switzerland). At the time of bypass, the segment of harvested external jugular vein was gently flushed of blood, stented, and fixed by a bridged microclamp. This arrangement allowed rotating the vein graft 180 degrees to provide a more uniform external irradiation of the tissue. Vein grafts were immersed in phosphate-buffered saline solution and externally irradiated ex vivo with the argon-pumped dye laser (Coherent INNOVA I 100 and Coherent CR599; Coherent, Palo Alto, Calif.) at 675 nm at a uniform irradiance of 100 mW/cm^2 for a total delivered fluence of 100 joule/cm^2.[19]

Harvest. A pilot study of PDT-treated vein grafts included six rats that were harvested at 24 to 96 hours. For the main study nine animals (five PDT VG and four CON VG) were killed at 2 weeks, and nine animals (four PDT VG and five CON VG) were killed at 4 weeks after surgery. Animals received bromodeoxyuridine (Sigma Chemical Co., St. Louis, Mo.) 100 mg/kg intramuscularly, at 18 and 12 hours before sacrifice. After anticoagulation with 1000 U heparin and euthanasia, the animals were infused with Hanks' solution at 80 mm Hg pressure, exsanguinated, and perfusion fixed with 10% buffered formalin. Specimens included vein grafts with adjacent artery and segments of small bowel and contralateral carotid artery, which served as immunohistochemistry controls.

The animal procedures were approved by the Institutional Animal Care Committee and complied with the "Principles of Laboratory Animal Care" and the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1985).

Histology. The harvested vein graft specimens were processed into three segments: proximal anastomosis, vein graft body, and distal anastomosis. Cross-sections were prepared from the vein graft body, whereas longitudinal sections were obtained from the anastomoses. Specimens were stained with hematoxylin and eosin, with Masson's trichrome for collagen, and Verhoeff's elastin stains. Intimal and medial areas were measured by morphometric analysis with a camera lucida digitizing measurement system (Sigma Scan; Jandel Scientific, Sausalito, Calif.).[23] The luminal stenosis was calculated as a percent of the intimal area over the total area within the internal elastic lamina area. The vein graft diameter was calculated from the circumference measured as the length of the internal elastic lamina. On longitudinal sections, IH was measured across

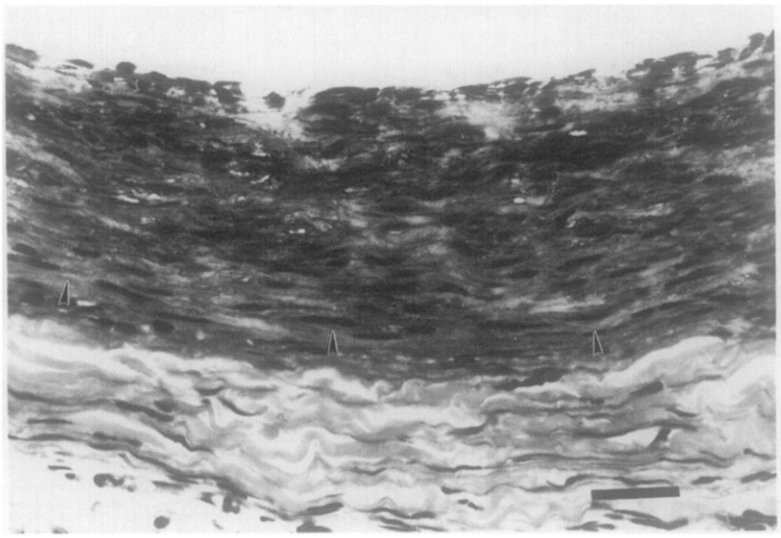

Fig. 2. Light micrograph of representative section from 4-week control vein graft. Note thin media and IH with multiple layers of smooth muscle cells. *Arrow* denotes internal elastic lamina. Hematoxylin and eosin stain. *Bar* equals 25 μm.

the anastomoses as thickness from the internal elastic lamina.

Immunohistochemistry. Four-micrometer–thick sections were deparaffinized through xylenes and rehydrated with graded alcohol. Specimens were prepared as previously described with the primary antibody HHF-35 (Biogenics, San Ramon, Calif.) for smooth muscle specific actin or bromodeoxyuridine (Cell Proliferation Kit; Amersham, Arlington Heights, Ill.) for detection of S-phase in mitosis to assess cell proliferation.[23] Bromodeoxyuridine-labeled nuclei were then counted in three random 400 X fields per specimen to assess the number of positive cells and compared with HHF-35–stained slides to identify proliferating SMC.

Statistical Analysis. All data within experimental groups were expressed as mean ± SEM. Differences between groups were evaluated where appropriate with either one-way analysis of variance or a Student *t* test. A p value less than 0.05 was regarded as significant.

RESULTS

All vein grafts were patent at harvest. Four animals, two PDT VG and two CON VG, died in the early phase of this study and were excluded from the analysis. No animals appeared to develop skin photosensitivity. Histologic study of the PDT VG in the pilot study demonstrated no acute thrombus formation. Microscopically, platelets but not polymorphonuclear leukocytes were seen on the intimal surface. Rare nuclei were seen in the vein graft media.

Eighteen rats were used for the main, long-term study. There was no evidence of graft dilation or aneurysm present in any vein grafts. At harvest there was less inflammation around the PDT VG, which were easier to dissect than CON VG.

Microscopically at 2 weeks after surgery, CON VG developed typical IH consisting of 8 to 10 layers of SMC with collagen and some elastin matrix (Fig. 2). Occasional polymorphonuclear cells could be seen in the CON VG adventitia. In the PDT VG (Fig. 3), IH was identified on an occasional specimen, but it consisted of only two to three layers of SMC. In contrast to CON VG, PDT VG had a significantly reduced adventitial inflammatory infiltrate. No substantial difference in structure between 2- and 4-week vein grafts was found within each group. Endothelial cells were noted in both CON VG and PDT VG sections. Anastomotic hyperplasia appeared equivalent in CON VG and PDT VG with SMC present primarily in the vein graft portion of the anastomoses (Fig. 4).

The results of morphometric analyses are shown in Table I. In PDT VG, there was a fivefold decrease of intimal area at 2 weeks and almost a twofold decrease at 4 weeks compared with corresponding CON VG ($p < 0.05$). The percent luminal stenosis reflected the findings of the intimal area (Table I). The medial area of PDT VG was also consistently less

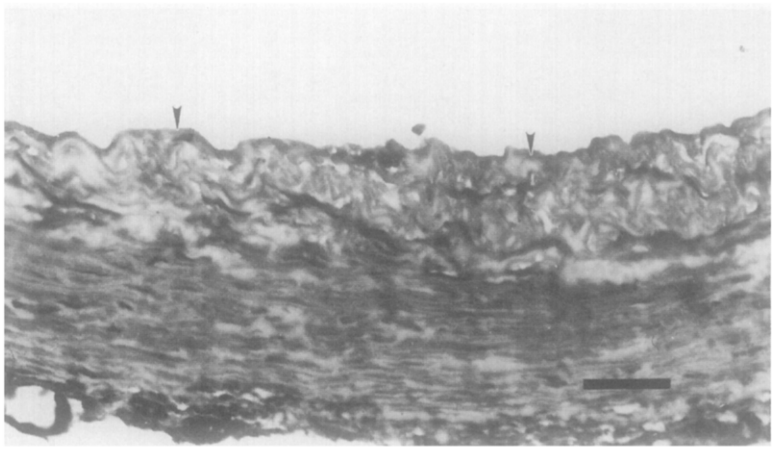

Fig. 3. Light micrograph of representative section from 4-week PDT vein graft. No IH is present. Note collapsed media and compact adventitia. *Arrow* denotes internal elastic lamina. Hematoxylin and eosin stain. *Bar* equals 25 μm.

Fig. 4. Light micrograph of 4-week anastomotic hyperplasia of PDT vein graft. Smooth muscle cells appear to be emanating *(arrow)* from artery wall edge with black elastic laminae into IH. Verhoeff's elastin stain. *Bar* equals 50 μm.

at 2 and 4 weeks compared with CON VG ($p < 0.05$). There was a significant decrease in CON VG diameter noted between week 2 and week 4 ($p < 0.05$). Although this trend existed in PDT VG, it was not statistically significant.

Anastomotic longitudinal morphometric analyses are shown in Figs. 5 and 6. IH was not characteristic for the arterial portion of the anastomoses except at the distal anastomoses of the 4-week PDT VG. There was no statistically significant difference between PDT VG and CON VG IH across the anastomoses at 2 and 4 weeks.

Immunohistochemistry with HHF-35 for SMC-specific actin verified that the IH present at the anastomosis and body of vein grafts consisted of SMC. CON VG had positive staining results in the media, whereas PDT VG had no cells in the media. Results of immunohistochemistry to delineate cell proliferation by bromodeoxyuridine-positive nuclei are listed in Table I. In all groups there was more evidence of cellular proliferation at 2 versus 4 weeks. There were no noted differences between CON VG and PDT VG bromodeoxyuridine labeling at the anastomoses; however, in the PDT VG body there

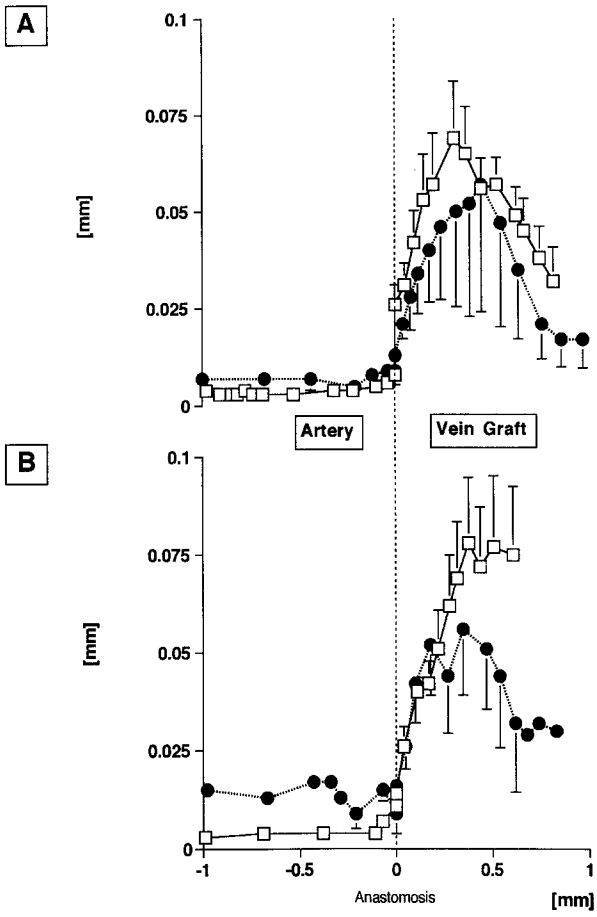

Fig. 5. Distribution of IH at proximal anastomoses. IH is measured at 2 weeks **(A)** and 4 weeks **(B)** between PDT *(closed circles)* and control *(open squares)* vein grafts at distances in mm.

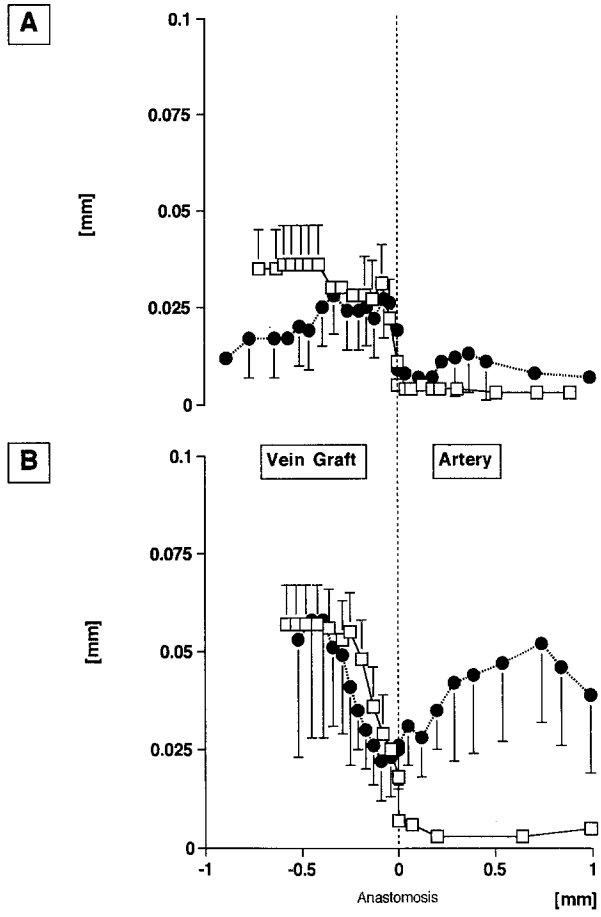

Fig. 6. Distribution of intimal hyperplasia at distal anastomoses. IH is measured at 2 weeks **(A)** and 4 weeks **(B)** between PDT *(closed circles)* and control *(open squares)* vein grafts at distances in mm.

was rare labeling and only in the adventitia. The immunohistochemistry verified that most of the proliferating cells in CON VG media and intima stained positive for SMC-specific actin.

DISCUSSION

The pathogenesis of vein graft IH, a significant problem in vascular surgery, is not fully understood. Vein graft IH may be a purely adaptive response to arterialization with limited cellular proliferation and matrix deposition. However, as an exaggerated response to injury, IH may play a pivotal role in the development of stenoses and clinical vein graft failures. In this study, we demonstrated that, when experimental vein grafts are subjected to PDT before implantation, the development of IH in the body of the graft is diminished, resulting in less luminal stenosis. However, anastomotic IH is not affected by this treatment. Therefore these data suggest that the origin of the SMC causing vein graft body and anastomotic IH may be different.

Previous results from this laboratory indicated that PDT can effectively prevent the development of IH in the balloon-injured rat carotid artery.[19,23] Initially acellular without evidence of an inflammatory reaction to the cytotoxic effects of PDT, the intima of PDT-treated arteries eventually develops endothelial cell covering as suggested by scanning electron microscopy.[23] Long-term studies demonstrated rare SMC repopulation of the media, and only the occasional PDT-treated artery developed IH by 16 weeks. Despite these findings there was neither artery dilation nor an increased compliance suggestive of weakening of the vessel wall.[23,27] These results, confirmed by others,[28] demonstrated favorable long-term healing of the artery after PDT. The data from

these arterial studies prompted this investigation with a similar PDT regimen used to determine the feasibility of IH inhibition in the vein graft model. A lower concentration of the photosensitizer CASPc was used because the relatively thinner venous wall was believed to facilitate drug diffusion and light penetration as compared with the artery.

The hypothesis of this study was that PDT depletion of cells from the vein graft and production of a stable collagen scaffold would prevent vein graft IH or dilation. In fact, the results of this investigation support this hypothesis and demonstrated many similarities with the PDT effects on arteries. PDT produced a hypocellular vein graft without inflammation in the vessel wall. Loss of endothelial cells in the vein graft after PDT did not cause increased thrombogenicity. Over time cell repopulation occurred in the intima and adventitia of PDT-treated vein grafts. IH was substantially reduced by PDT as compared with CON VG in the body of the graft.

IH in vein grafts has been reported, in part, to be a result of the adaptation to the tangential stress experienced by the vessel wall during exposure to arterial circulation.[29] In the cited study, which used a rabbit model, intimal areas and diameters continued to increase over the 24 weeks examined. However, our data reflected a stabilization of CON VG intimal and medial areas and a decrease of the diameters at the 2- and 4-week time points examined. This may reflect the different design of this study, which provided similar sized animals at the time of euthanasia to correct for possible growth differences. The decrease of the CON VG diameters, which were isometric with the adjacent artery at the time of implantation, may reflect vein graft fibrosis. Vein grafts treated with PDT may not contain the necessary cells to produce the fibrosis, which resulted in the diameter reduction noted at 4 weeks in CON VG.

Photodynamic therapy did not affect anastomotic vein graft IH in this model. Despite cell depletion in the PDT VG, the histologic structure, cell proliferation, and amount of IH at the anastomoses were virtually the same compared with CON VG at 2 and 4 weeks. Immunohistochemistry confirmed these anastomotic IH cells of PDT VG and CON VG to be SMC.

Compliance mismatch may be a contributing factor to the anastomotic IH noted in both PDT and CON VG. Reported increased compactness of the collagen in the adventitia of PDT-treated arteries occurred over time and leads to a decrease in dynamic compliance of the artery wall as compared with non-PDT-treated arteries.[27] The degree of compliance mismatch present at the anastomosis has been reported to affect the amount of IH that develops primarily beyond the distal anastomosis.[30,31] Although compliance of these vein grafts or anastomoses were not evaluated, the inferred compliance differences may explain the presence of IH found in the artery at the distal anastomosis in the 4-week PDT VG.

Studies have established that SMC can migrate from the arterial wall to the vein graft to produce IH in response to surgical injury.[8-10] Because our results demonstrated the absence of cells in the PDT VG acutely after treatment, the presence of anastomotic IH would indicate that the SMC responsible for the changes were of arterial origin. In fact, increased bromodeoxyuridine labeling of nuclei, indicative of proliferating cells, in the arterial media adjacent to and at the anastomosis supports this hypothesis.

In conclusion, PDT suppressed vein graft IH, which may have a role for inhibiting stenoses of the vein graft body. However, PDT of the vein graft alone could not suppress anastomotic IH. This may imply a different cause of the stenosis of the vein graft body compared with the stenosis at the anastomotic site. Anastomotic vein graft IH may result from arterial rather than venous injury or arterialization. Therefore further investigations need to be undertaken to determine whether PDT treatment directed to the anastomosis can inhibit IH at those sites.

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DISCUSSION

Dr. Steven Ruby (Farmington, Conn.). IH and its direct consequences—recurrent stenosis and vein graft occlusion—plague us as vascular surgeons on a daily basis. Our colleagues from the Massachusetts General Hospital have presented an elegant experiment as part of their relentless assault on this perplexing problem. The uniform response of blood vessels to injury has been catalogued in detail but is yet to be completely understood. The initiating events may be a combination of mechanical, biochemical, physiologic, and immunologic factors. As a result, experimental and clinical interventions have been diversified. The goal of this experiment was to assess the role of PDT in the pretreatment of interposition vein grafts before their implementation in a rat model, and the answer to this question was partially yes. The good news is that IH in the body of the graft was essentially eliminated. The bad news is that where it really counts clinically at the Achilles' heel of what we do at the distal anastomosis, this application of photobiology was not effective because the origin of the pathologic process presumably was the adjacent artery or

the result of an accentuated compliance mismatch. The results were intriguing, and they raise more questions than they answer.

There was a virtual elimination of endothelial cells as a result of pretreatment with laser light, yet there was no acute thrombosis of the vein grafts. Are we to believe that it is no longer advantageous to preserve healthy functioning endothelial cells to maintain functioning vein grafts? Why did the exposed collagen not result in thrombosis?

In this work and in all prior work with this model of PDT, there has been a virtual elimination of all inflammatory cells in the wall of the vessel. Like anything else, too much inflammation is probably a detriment; however, what will the ultimate healing of these grafts be like in the absence of any inflammatory infiltrate?

In the manuscript you briefly touch on the compliance change induced by the laser treatment. Will this ultimately be the stumbling block? You said the vein was thin and blue, but on the other hand you also stated that the compliance was decreased, so maybe you could just readdress that for us.

From a technical point of view, is there a difference between external irradiation of the grafts as has been done in this experiment and the internal application of light? If you change the dose or the color of the light, will you preserve more of the endothelial cells?

Last, what are the clinical implications? We would have to conclude from this study that just excising the graft before implantation and bringing it to the laboratory for irradiation will not help with the IH at the distal anastomosis. What then are the practical considerations, and how can we now aim our attack with this technology on the SMC of the adjacent artery?

Dr. Glenn M. LaMuraglia. From our previous work in arteries, we have known that thrombosis is not a problem if the proper dose of PDT is used. Many things happen with free radicals that we are not sure of. We are starting to investigate some of these possibilities, changes in the artery wall so that the surface may be less thrombogenic than it is in other circumstances such as mechanical injury to the subendothelium or the basement membrane.

In terms of the lack of inflammation, this is the most important finding and why we have continued with this work. Without inflammation and protein turnover in the vicinity of white cells, there is no weakening in the wall. As a result it permits cellular repopulation, as previously described, into the adventitia to enable a scaffolding around the artery and to help prevent the development of aneurysmal degeneration. Therefore, besides the adventitial healing, although we have not presented it, we also expect the endothelium to repopulate the intima of the vessel as we have described in our arterial model.

Your question regarding compliance is in fact correct. These vessels do become stiffer over time, which probably relates to the adventitial healing process that occurs in these vessels over weeks and months. It is detrimental; however, there are ways of grading such a phenomenon, and one of the possibilities is in applying the PDT to adjoining arterial surfaces.

In terms of the questions relating to the external versus the internal laser irradiation. The size of these vein grafts, approximately 1 mm in diameter, makes it essentially an engineering feat to try to irradiate from the inside. We have used 675 nm wavelength light, which is in the red part of the spectrum and has a very deep penetration in tissue approximately 1 to 2 mm. These vein grafts were irradiated in vitro and were moved during the irradiation time to provide a homogeneous irradiation. We were testing more the principle than a possible clinical application at this early phase.

There is no wavelength of light to deplete all the SMC and not the endothelial cells. However, as you know, other options exist such as endothelial seeding of grafts if these are going to be prepared in a laboratory and then placed into patients.