Inhibition of In-stent Restenosis in Rabbit Iliac Arteries with Photodynamic Therapy

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Objectives. Photodynamic therapy (PDT, the combination of light with a photosensitising drug in the presence of oxygen) inhibits restenosis after angioplasty without stenting. This study assesses the potential of PDT for prevention of in-stent restenosis.

Design and methods. Normal rabbits were given the photosensitising agent 5-aminolaevulinic acid (ALA) 60 mg/kg, 3 h prior to endovascular illumination of the iliac artery (635 nm at 50 J/cm²) either immediately before or after deployment of an oversized (3 mm diameter) stent. PDT treated arteries were retrieved 3 or 28 days later and assessed for cell counts and vascular morphometry. Control arteries (stent but no PDT) were examined at 28 days.

Results. There were no adverse events and all vessels were patent at the end of the study. At 3 days there was almost complete medial cell ablation when light was delivered before stent deployment (17 ± 1 cells/hpf), with little effect when illumination followed stent deployment (184 ± 17 cells/hpf, p < 0.0001). Twenty-eight days after PDT, the neointimal areas were 1.41 ± 0.52 mm² (stent with no PDT), 1.24 ± 0.54 mm² (light after stent) and 0.60 ± 0.21 mm² (light before stent) (p = 0.004).

Conclusions. PDT before stent deployment caused almost complete medial cell ablation at 3 days with inhibition of in-stent restenosis at 28 days. PDT is worthy of further study as an adjuvant to percutaneous intervention in patients with vascular disease.

Keywords: In-stent restenosis; Photodynamic therapy.

Introduction

Vascular smooth muscle cell (VSMC) proliferation with migration into the intima is a major contributor to the development of restenosis after angioplasty or stent deployment. No systemic pharmacological agent has yet been shown to resolve this problem. Most current interest centres on techniques that alter cell proliferation, either by elution of a cytotoxic drug from the stent wall or by brachytherapy.1 An alternative approach to regulation of VSMC proliferation is photodynamic therapy (PDT). PDT produces local cell killing with low power red light after the prior administration of a photosensitising drug and in the presence of oxygen.2

Several photosensitising agents have been studied in relation to arterial disease, particularly motexafin lutetium and 5-amino-laevulinic acid (ALA, which is converted in vivo into the photoactive derivative, protoporphyrin IX, as part of the chemical chain for the synthesis of haem). PDT experiments on normal arteries have demonstrated endothelial denudation, which heals in a few days, and medial cell depletion, which recovers over a much longer period, but with maintenance of the vessel integrity and strength at all times after treatment. There has been no evidence of aneurysm formation or thrombosis.3-5 Further studies have shown that PDT can inhibit neointimal hyperplasia following angioplasty to rat carotid, rabbit iliac and pig coronary arteries.6-9 Pilot safety studies of its application to peripheral and coronary disease in man have now been reported.10-13

About 80% of coronary angioplasties are now stented. These vessels may re-occlude by neointimal hyperplasia brought about by a number of different processes including VSMC proliferation, causing in-stent restenosis.14,15 The combination of PDT and arterial stenting has not yet been studied in detail. The aim of the present study was to address this...
question, in particular to examine how ALA-PDT affects neointimal hyperplasia when given before or after stenting rabbit iliac arteries.

Methods

Photosensitiser

The photosensitising agent used was 5-aminolaevulinic acid (ALA, supplied as the hydrochloride, DUSA Pharmaceuticals, USA). This was dissolved in normal saline and buffered to pH 7 with sodium bicarbonate. This was administered intravenously at a dose of 60 mg/kg 3 h before light delivery.3

Animals and surgical techniques

New-Zealand white rabbits (n = 32, 2.5–3 kg) were used in this study. The rabbits were anaesthetised using Hypnorm (fentanyl/fluanisone) 0.5 ml/kg (Janssen), and Hypnovel (midazolam) 0.05 ml/kg (Roche) for induction and maintained with inhalation anaesthesia using halothane. Following skin preparation, the superficial femoral artery was exposed and bathed externally in papaverine (Martindale, Romford) for 2 min. An arteriotomy allowed insertion of a 2F canula and an angiogram delineated the anatomy of the aorta and the iliacs. Images were obtained using a C arm image intensifier with a road mapping facility (Siemens S2000, Erlangen, Germany), which allowed accurate intravascular positioning and recorded on super VHS video tape. At the end of stent and light delivery the superficial femoral artery was ligated and the wound repaired. The rabbits were recovered and maintained on standard rabbit chow and daily aspirin (20 mg/kg). All animals were treated in accordance with the Animals (Scientific Procedures) Act 1986 under project and personal licences approved by the Home Office (UK Government). The investigation conforms with the ‘Guide for the Care and Use of Laboratory Animals’ by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Stenting and light delivery

A specially constructed transparent balloon catheter was used for endovascular illumination. (Occam, The Netherlands). This had a 20 mm by 3 mm balloon with a blind ending channel into which was pre-loaded an optical fibre (0.2 mm core and a 20 mm distal radial light diffuser of diameter 0.5 mm), with radio opaque markers. The catheter was introduced over a monorail guide wire into the ipsi-lateral common iliac artery so that the balloon lay in the target segment just proximal to the iliac bifurcation. The wire was pulled back from the balloon when the correct position was obtained. During light delivery the balloon was inflated with air sufficiently to occlude the blood flow but not to overstretch the vessel. Red light was delivered from a diode laser (Diomed, Cambridge UK) at 635 nm, 80–100 mW/cm² of diffuser with a total dose of 50 J/cm² on the surface of the balloon. The total light delivery time in each procedure was 5–600 s, excluding a 60 s pause after 1 min of light delivery. Such a pause during illumination has previously been shown to increase the efficacy of PDT with ALA.16,17 After light delivery the deflated balloon, guide wire and optical fibre were removed. Either before or after light delivery, a 15 mm by 3 mm stent was loaded on a 3 mm balloon catheter, introduced through the femoral arteriotomy, positioned under X-ray guidance and deployed at the target site at 10 atm. pressure.

Animals were randomly assigned to one of three groups:

Group 1: (n = 8): Stent deployed without drug or light application.
Group 2: (n = 12): ALA given 3 h before surgery with light delivery for PDT after stent deployment.
Group 3: (n = 12): ALA given 3 h before surgery with light delivery for PDT before stent deployment.

Tissue harvesting and processing

All rabbits were killed by administration of an overdose of 20% phenobarbiturate. The abdomen was opened, the retroperitoneum exposed, and the aorta isolated and tied above its bifurcation. An 18F IV canula was introduced into the distal aorta and the distal vessels were pressure perfused at 100 mmHg with 4% formalin for 10 min. The iliac arteries were then isolated and removed and fixed for a further 16 h in 4% formalin.

Four rabbits in groups 2 and 3 were culled at 3 days. In these animals the stented vessels were isolated, cut open along the longitudinal axis and peeled from the stent. The untreated contra lateral iliac arteries were used as controls. The harvested tissues were embedded in paraffin wax, cut into 4 µm sections and stained with haematoxylin and eosin (H & E). Photomicrographs of these sections were taken (JVC TK-1281) and transferred onto a personal computer for morphometric analysis using the Lucia-M (version 3.52a) programme. Three sections from each vessel.
were examined. On each, the condition of the endothelium was documented and the number of VSMC nuclei per high power field (20×objective) was counted in 10 fields in all quadrants to calculate a mean count per high power field.

The other rabbits (eight in each of the three groups) were culled at 28 days after stenting and light delivery. These vessels were embedded in LR white resin using standard techniques with the stent left in situ. Vessels were cut into 10 μm sections with the stents in situ on a tungsten microtome and stained subsequently with H & E.

Morphometric analysis included measurement of the luminal area, the area within the internal elastic lamina (IEL), the area within the external elastic lamina (EEL), and the intimal thickness. The intimal area was calculated by subtracting the luminal area from the area within the IEL. Similarly the medial area was calculated by subtracting the area within the IEL from the area within the EEL. The best section, i.e. one that was intact and whole, from each vessel was used for measurement. Intimal thickness was measured at 10 different points around the artery circumference, at approximately equal separations. The mean of these 10 measurements was used in the subsequent analysis.

![Graph showing Vascular smooth muscle cell (VSMC) counts in group 2 and 3 at 3 and 28 days after PDT compared to cell counts in the normal rabbit artery media showing significant ablation in group 3 at 3 days. (p < 0.005 group 2 vs. group3) there is partial repopulation of the media in group 3 at 28 days.](image)

**Fig. 1.**

Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was by the comparison of the means from each group using the unpaired Student t-test. Inter and intra-observer variability was assessed by reanalysis of a representative sample of histological sections. Area and thickness calculations were made by one observer on two separate occasions and by a second independent observer on one occasion. There was no significant difference in the inter and intra-observer readings. A p value of <0.05 was considered significant.

Results

All animals survived to the end of the experimental period and there was no evidence of thrombosis, rupture or aneurysm formation in either of the PDT treatment groups or in the control group.

The media cell counts 3 days and 28 days after stenting and light delivery are shown in Fig. 1. Light treatment before stenting significantly reduced VSMC in the media at 3 days, whereas light treatment after stenting did not. (Fig. 2(a) and (b)). In neither group were endothelial-like cells seen on the luminal surface.
of the retrieved arterial fragments from which the stent had been removed prior to processing. Observations of the sections of the resin-embedded stented arteries at 28 days showed that there was partial repopulation of the media in group 3 while the other two groups retained their cellularity throughout. (56 ± 7 counts per high power field in group 3, 155 ± 14 in group 2 and 167 ± 13 in group 1). Representative samples are shown in Fig. 2(c)–(f).

The morphometric measurements 28 days after stenting are shown in Table 1. By this time, the stented arteries had all developed a neointimal layer, but in group 3 (PDT light before stent deployment), this was significantly thinner than in the other two groups. There was no statistical difference in either the area within the EEL or the area within the IEL confirming that deployment of the stents was to a similar diameter in all groups with no expansile remodelling in PDT treated groups. The percentage stenosis was calculated as the ratio of the neointimal area to the area within the IEL and was significantly less in group 3 than in either of the other two groups (Fig. 3).

While no immunohistochemical confirmation of endothelial integrity was possible with the LR white resin mounted tissue, a single layer of flat endothelium-like cells lined the stented arteries at 28 days in all groups (Fig. 2(g) and (h)).

**Discussion**

This study is the first to demonstrate that PDT can be an effective adjuvant to arterial stenting, significantly reducing intimal hyperplasia, without detrimental effects on vascular integrity and thrombogenicity. However, activation of photosensitiser must take place before rather than after the stent is deployed.

We used the rabbit iliac artery, a well-established model for in-stent restenosis, although these are disease-free, elastic arteries. There may be a difference in response when PDT is applied to atherosclerotic vessels with the addition of both cellular elements such as macrophages and fibroblasts and a lipid core. Previous studies have shown the *in vivo* accumulation of photosensitisers in atherosclerotic plaques both in the cellular and non-cellular elements, although this has not been reported using ALA.20,21 Nevertheless, rabbit models of atherosclerosis are quite different from human atherosclerotic lesions and do not mimic complex human coronary disease. We considered that the model we used was appropriate for answering the key question of how PDT before and after stenting influenced cell proliferation. Our studies did only extend to 28 days, but this is a time point frequently used in preclinical studies.22 Later observations might have revealed a catch up of the lesion, but we think this unlikely as our previous studies following PDT treated arteries in rats for up to 6 months did not show any marked later cell proliferation.8

In general, the response of tissue to PDT is complex, with inflammation and microvascular damage, but in the arterial wall the development of medial apoptotic bodies, TUNEL staining of residual nuclei and the lack of an early inflammatory infiltrate suggest that the predominant effect is apoptosis.23–25 As documented for PDT as an adjuvant to angioplasty without stenting, the prevention of intimal hyperplasia and restenosis seen in this study is linked closely to

**Table 1. Morphometric measurements at 28 days after PDT in all groups**

<table>
<thead>
<tr>
<th></th>
<th>Stent only controls (group 1)</th>
<th>Light delivery after stenting (group 2)</th>
<th>Light delivery before stenting (group 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area within EEL (mm²)</td>
<td>5.47 ± 1.05</td>
<td>5.81 ± 1.33</td>
<td>5.76 ± 1.32</td>
</tr>
<tr>
<td>Area within IEL (mm²)</td>
<td>5.05 ± 0.92</td>
<td>5.28 ± 1.15</td>
<td>5.30 ± 1.25</td>
</tr>
<tr>
<td>Luminal area (mm²)</td>
<td>3.64 ± 0.40</td>
<td>4.04 ± 0.67</td>
<td>4.71 ± 1.07†</td>
</tr>
<tr>
<td>Intimal area (mm²)</td>
<td>1.41 ± 0.52</td>
<td>1.24 ± 0.54</td>
<td>0.60 ± 0.21†</td>
</tr>
<tr>
<td>Intimal thickness (mm)</td>
<td>0.90 ± 0.36</td>
<td>0.88 ± 0.29</td>
<td>0.47 ± 0.13†</td>
</tr>
<tr>
<td>Medial area (mm²)</td>
<td>0.42 ± 0.15</td>
<td>0.53 ± 0.33</td>
<td>0.46 ± 0.24†</td>
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* Statistically significant compared to group 1 (p<0.05).
† Statistically significant compared to group 1 and 2 (p<0.007).
ablation of cells in the media. There is repopulation of the media of pig coronaries illuminated endovascularly, consistent with the partial medial cell repopulation at 28 days documented here, although cell depletion may persist for 6 months or more in rat carotid arteries. In addition to the apoptosis, it has been suggested that the lack of cell migration after PDT reflects local PDT-induced cross-linking of matrix proteins, which renders the interstitium resistant to proteolytic digestion by migrating cells.

The striking result from this study is the observation that the media remained fully populated with smooth muscle cells with no inhibition of stent-induced intimal hyperplasia at 28 days if the light was delivered after stent deployment. There are several possible explanations. As the ALA was given at the same time prior to light delivery in both groups 2 and 3, the difference in response is unlikely to be related to the distribution of protoporphyrin IX (the photoactive derivative of ALA). PDT requires oxygen. The simplest explanation is that stent deployment stretches the arterial wall and makes it hypoxic. It has been documented previously in the rabbit that deployment of an oversize stent renders the normal arterial wall hypoxic. This effect is most marked in the media. Oxygenation of the endothelial layer is maintained better from blood flowing through the lumen of the vessel.

stent insertion, although this loss may be at least partially attributable to the trauma of the balloon-mounted stent delivery. By 28 days all stented arteries had a layer of endothelial-like cells coating the lumen surface of the artery. Several studies report complete and even accelerated regrowth of normal vascular endothelium after PDT without stenting, with no evidence of thrombosis or any other impairment of blood flow. Early re-growth of endothelium after PDT may play a role in the inhibition of VSMC proliferation and intimal hyperplasia and may also play a role in reducing thrombogenic complications after stent placement.

There are other possible explanations for our results. The vascular stent is a metal tube mesh, so optical shadowing from light delivered from within the lumen of the stent must occur to some degree. However, the wire of the stent struts is so thin compared with the penetration depth in tissue of the red light used (typically about 2 mm) that scattering of light within the tissue should make this a minor consideration. Nevertheless, a recent report describes inhibition of intimal hyperplasia from PDT given after stenting of the femoral artery, but employing illumination of the external surface of the artery, perhaps avoiding strut shadowing. Another possibility is that mechanical stretching of the arterial wall by the stent could lead to on-going stimulation of VSMC proliferation (in contrast to the single insult of a balloon.

Fig. 3. Histogram showing percentage stenosis (ratio of intimal area to area within IEL) in groups 1, 2 and 3 at 28 days. (p<0.001, group 1 vs. group 3, p<0.002, group 2 vs. group 3).
angioplasty followed by removal of the balloon. However, this is less likely as the effect would have been expected to be the same in both PDT treated groups.

Most knowledge of the arterial effects of PDT comes from experiments on arteries in normal animals, but there are now a few reports of clinical applications. The first study, reported in 1999, described seven patients in whom eight lesions had restenosed after a previous femoral artery angioplasty. All were asymptomatic with patent vessels 6 months after repeat angioplasty with adjunctive ALA-PDT.\(^\text{10}\) A 4 year follow up on the same patients showed that only one of eight treated sites showed any evidence of restenosis, despite the absence of any anti-thrombotic treatment other than aspirin.\(^\text{10,12}\) A randomised study of femoral angioplasty with and without adjuvant PDT is currently under way. Motexafin lutetium has also been tested in clinical trials of peripheral and coronary vascular disease\(^\text{13}\) including a phase 1 study as an adjuvant to stenting in coronary arteries. The safety and tolerability were excellent and the late lumen loss and binary restenosis rate were at least as good as after bare stenting.\(^\text{11}\) Nevertheless, the study was not designed to assess the efficacy of adjuvant PDT, which will require further studies.

The potential value of PDT as an adjunct to balloon angioplasty compared with other current options such as the elution of cytotoxic drugs from stents and brachytherapy, will depend partly on the cost and convenience of treatment delivery, but more importantly on the long term biological effects on the arterial wall. The key role of medial VSMC in the intimal response to a stent is reflected in the significant reduction in neointimal area and thickness at 28 days only under conditions where PDT depletes the VSMC at 3 days. This correlation is also seen with drug eluting stents and brachytherapy.\(^\text{1,32,33}\)

Remodelling with vessel shrinkage is a major contributor to restenosis after balloon angioplasty. Previous studies from our group and others have shown that following angioplasty with adjuvant endovascular PDT but without stenting, there is inhibition of constrictive remodelling.\(^\text{34,6}\) Remodelling is less important in the presence of a stent, but it is reassuring that we saw no evidence of aneurysmal dilatation following PDT, nor signs to suggest stent mal-apposition, which has been a feature of both brachytherapy and drug eluting stents.\(^\text{35,36}\) Further, no signs of injury to adjacent tissue have been reported with ALA-PDT, which may reflect the limited tissue penetration (2 mm) of the 635 nm light used to activate protoporphyrin IX, the active metabolite of ALA. The impaired endothelial re-growth after brachytherapy and drug eluting stents has necessitated long term anti-thrombotic treatment with the associated expense and risk of bleeding complications.\(^\text{37–39}\) The rapid re-endothelialisation reported after PDT in animals allows us to speculate that in the clinical setting adjuvant PDT may require less subsequent maintenance drug therapy. The endothelium may not heal so well in atherosclerotic human vessels as it does in non-atherosclerotic animal arteries, but the one clinical study so far reported with extended follow up of 4 years, did not describe any evidence of late thrombotic complications despite only aspirin prophylaxis.\(^\text{12}\) A recent report on long term follow up of drug eluting stents described four cases in which drug eluting stents in coronary arteries thrombosed more than a year after insertion, when anti-thrombotic therapy was reduced.\(^\text{40}\) Two of these patients also had non drug eluting stents, which did not thrombose. There were no thrombotic complications in our experiments. On the evidence currently available from this and other studies, arteries tolerate PDT remarkably well, in the short and long term, although the clinical data available is very limited. No reports of arterial PDT have described any evidence of the chronic changes known to be associated with ionising radiation.

One of the key attractions of PDT is the nature of the biological effect. Despite its increasing use in a range of specialities, there is no evidence of cumulative toxicity and it has often been used to treat the same site several times. The photon energy of the red light used is too low to damage DNA. There is a lack of any effect on the mechanical integrity of arteries or on the blood flow through them and a lack of any chronic changes detectable histologically. This body of evidence makes PDT an attractive option as a possible alternative to drug eluting stents and brachytherapy. However, as PDT has only been shown to work if the light is given prior to stent deployment, it may not be of value for the treatment, rather than prophylaxis, of in-stent restenosis. The published data on stent induced hypoxia in the wall of arteries showed that there was some improvement in the hypoxia when the stents had been in situ for several weeks, but further detailed experiments would be required to see if PDT can have any value for treating rather than preventing in-stent restenosis.

In practical terms, PDT is more complex than inserting a drug eluting stent, but with the design of balloon catheter described here, it is straightforward to deliver the light intraluminally. The need to administer ALA several hours prior to angioplasty and to take simple precautions against exposure to bright ambient light for 1–2 days are relatively minor inconveniences.
The safety precautions required are certainly less than are needed for brachytherapy.

On the limited evidence available to date, it would appear that PDT may be able to reduce the incidence of re-stenosis after angioplasty, with or without stenting, with few, if any, long term adverse effects on the arterial wall. Further studies are needed to clarify the mechanisms involved, particularly in stented arteries, and to undertake larger scale clinical trials.

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