Photoangioplasty with local motexafin lutetium delivery reduces macrophages in a rabbit post-balloon injury model

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Abstract

Objective: Motexafin lutetium (Lu-Tex, Antrin\textsuperscript{®} Injection) is a photosensitizer that selectively accumulates in atheromatous plaque where it can be activated by far-red light. The localization and retention of intra-arterially administered Lu-Tex and its efficacy following activation by endovascularly delivered light (photoangioplasty) was evaluated. Methods: Bilateral iliac artery lesions were induced in 17 rabbits by balloon denudation, followed by a high cholesterol diet. Lu-Tex distribution within the atheroma was examined (n=8) following local injection. Fluorescence spectral imaging and chemical extraction techniques were used to measure Lu-Tex levels within the atheroma and adjacent normal tissue. Photoactivation was performed 15 min following Lu-Tex administration (180 J/cm\textsuperscript{2} fiber at 200 mW/cm fiber). Two weeks post photoangioplasty, vessels were harvested and hematoxylin and eosin (H&E) and RAM11 (macrophages) staining was performed. Results: Local delivery of Lu-Tex achieved immediate high concentrations within plaque (mean 40\% control iliac atheroma). Mean percent plaque area in the treated segments was significantly lower than in the non-treated contralateral lesions (73 vs. 82\%, \textit{P}<0.01). No medial damage was observed. Quantitative analysis using RAM11 positive cells revealed significant reduction of macrophages in treated lesions in both the intima (5 vs. 22\%, \textit{P}<0.01) and in media (8 vs. 23\%, \textit{P}<0.01) compared to untreated contralateral segments. Conclusions: Local delivery provides high levels of Lu-Tex selectively within atheroma. Photoactivation results in a significant decrease in macrophage and a small decrease in atheroma burden without damage to the normal vessel wall. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Angioplasty; Arteries; Atherosclerosis; Macrophages; Restenosis

1. Introduction

Atherosclerosis is the leading cause of death in western society [1]. Fatty streaks begin with the deposition of cholesterol and infiltration of macrophages [2]. As the intimal thickening progresses, plaque rupture and erosion can lead to thrombus formation, leading to unstable angina or acute myocardial infarction. These culprit lesions are often angiographically silent and the luminal area is often well maintained due to vessel remodeling [3]. The pathobiology of these vulnerable plaques is currently being investigated with ample evidence suggesting that both macrophages and metalloproteases play crucial roles in such instability [4]. When the vessel is no longer able to compensate for the growing atheromatous plaque, luminal narrowing ensues, leading to myocardial ischemia. Mechanical endovascular interventions such as balloon angioplasty, stents, and atherectomy are all aimed towards enlarging the narrowed lumen but are not aimed towards retarding intimal growth or stabilizing vulnerable plaques [5–7].

The use of photoangioplasty using the photosensitizer, motexafin lutetium (Lu-Tex, Antrin\textsuperscript{®} Injection, Pharmacyclics, Inc., Sunnyvale, CA), has previously been

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reported in a hyperlipidemic rabbit model of atherosclerosis [8]. Lu-Tex is a water soluble, expanded porphyrin molecule with an absorption band centered at 732 nm, a wavelength of light that is capable of deep penetration through tissue and blood, and exhibits a fluorescence emission band at 750 nm. Lu-Tex has a plasma half life of 7 h following intravenous administration in humans [9]. Animal studies have demonstrated that this sensitizer binds lipoproteins and is transported into plaque, where it appears to localize in macrophages [8,10,11]. Regression of plaque, occurring over days, has been seen following light activation of the photosensitizer. In those studies, Lu-Tex was given intravenously and was activated by light delivered endovascularly using an optical fiber catheter. This modality, known as photoangioplasty, is based on the production of singlet oxygen by the light activated sensitizer, which destroys biomolecules and causes cytotoxicity [8].

In this study, we report the use of a modified Lu-Tex photoangioplasty technique for the treatment of atheromatous plaque in a rabbit post-balloon injury model. Lu-Tex is delivered locally rather than intravenously in order to minimize systemic exposure and to maximize local concentration. We investigated the effect of photoangioplasty treatment on atheroma burden and macrophage infiltration.

2. Methods

2.1. Photosensitizer

The synthesis and chemical characterization of Lu-Tex has formerly been described [12]. Its chemical structure and spectral characteristics are shown in Fig. 1. Lu-Tex exhibits absorption in the blue and infrared spectral regions, possessing extinction coefficients of 47 000, 126 000 and 42 000 M/cm at wavelengths of 414, 474 and 732 nm, respectively. Lu-Tex has a fluorescence emission peak centered at 750 nm following excitation at 470–480 nm. This fluorescence emission serves as a specific marker for the sensitizer since no endogenous chromophores emit fluorescence in this wavelength range. The Lu-Tex was formulated in 5% mannitol at a concentration of 2.3 mg/ml (2 mM).

2.2. Bilateral iliac balloon denudation model

Male New Zealand white rabbits, weighing approximately 3.5 kg, were maintained in Stanford University’s Department of Comparative Medicine in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were fed an atherogenic diet containing 1% cholesterol (Dyets, Bethlehem, PA). After 2–3 weeks, the rabbits underwent bilateral iliac balloon de-endothelialization. Each rabbit was anesthetized using an intravenous mixture of ketamine (5 mg/kg) and xylazine (35 mg/kg). A 5-Fr sheath (USCI, Billerica, MA) was inserted into the right carotid artery and into the descending aorta under fluoroscopic guidance. A 3.0×20 mm angioplasty balloon (Ranger, SCIN4ED, Maple Grove, MN) was advanced into either the right or left iliac artery. The balloon was inflated, with a 50% mixture of contrast and saline, and pulled back distal to the internal iliac artery three times at 8 atm. The same procedure was repeated in the contralateral iliac artery. Animals were maintained on an additional 6 weeks of atherogenic feeding prior to being recruited into the various study groups.

2.3. Lu-Tex biodistribution study

The left carotid artery was exposed, carefully incised and a 6-Fr sheath (USCI, Billerica, MA) inserted into the descending aorta under fluoroscopic guidance. A local delivery balloon catheter (3 mm, Dispatch, Scimed, Maple Grove, MN) was advanced to either the left or right iliac artery. For reference, the proximal end of the delivery catheter was placed, using fluoroscopic guidance, at the internal iliac artery. The balloon was inflated to 6 atm and LuTex (1.2 mg/kg) was infused at a rate of 0.2 ml/min over approximately 12 min. This dose was chosen as is one tenth the systemic dose previously used (12 mg/kg) [11]. Animals were euthanized and both iliac arteries and a portion of the lower abdominal aorta were harvested ‘en block’ immediately, 15 and 30 min post local Lu-Tex administration (n=2 in each group). An untreated group served as controls. Prior to sacrifice, plasma samples were prepared from EDTA anticoagulated arterial blood. Samples were stored at −80°C until fluorescence analysis was performed.
2.4. Fluorescence spectral bioimaging

Fluorescence spectral bioimaging was performed on the iliac arteries and lower abdominal aorta. Each aorta and contiguous iliac arteries were excised, cut longitudinally to expose the luminal surface and washed thoroughly with isotonic saline. The luminal surfaces of the vessels were inspected and atheromatous and adjacent normal areas were compared. The vessels were illuminated with an xenon lamp (Cogent Light Technologies, Inc., Santa Clara, CA) coupled to a 470-nm interference filter (10 nm bandwidth, Oriel Corporation, Stratford, CT). Images were collected with the 5D200 spectral bio-imaging system (Applied Spectral Imaging, Carlsbad, CA). A 715-nm long pass filter was utilized (Oriel Corporation, Stratford, CT) with capture of a fluorescence emission range of 650—850 nm. Each signal was averaged over 5 pixels. Each acquired measurement was imaged with a CCD camera coupled to an interferometer, and then the signal Fourier transformed allowing spectral identification at every pixel [13].

2.5. Quantitative Lu-Tex analysis

The right and left iliac arteries and the lower abdominal aortic segment were separated. The resultant tissue samples were weighed, frozen in liquid nitrogen and then pulverized using a stainless-steel pulverizer chilled to −40°C. The powdered material was homogenized (Polytron® (Brinkman) in 1.6 ml phosphate buffer (50 mM, pH 8.0) and then mixed thoroughly with 3.0 ml methanol. Chloroform (3.0 ml) was added and the resultant mixture shaken on a Thomas Shaking Apparatus (Arthur H. Thomas, Philadelphia, PA). The phases were separated by centrifugation (10 000xg, 10 min, room temperature). The chloroform-rich bottom phase was removed and brought to a volume of 3 ml with methanol. Lu-Tex levels were measured in tissue samples and plasma using a fluorescence assay [14]. Plasma samples (125 μl) were mixed with 10 mM Triton X-100 and also analyzed for Lu-Tex content using the fluorescence assay. Lu-Tex levels were expressed as μg sensitizer per g of tissue (wet weight) or μg sensitizer per ml of plasma.

2.6. Photoangioplasty

Endovascular illumination was performed by means of a fiberoptic catheter with cylindrical diffusing fibers at the distal end (TheraStat™ slim line, Laserscope, San Jose, CA). The cylindrical light diffusing portion of the fiberoptic measured 3 cm in length and 0.9 mm in diameter; radio-opaque gold markers are located at the distal and proximal end of the diffusing portion of the fiberoptic. Lu-Tex was photoactivated with 736 nm laser light that was produced with an argon pumped dye laser (Lambda Plus, Coherent, Palo Alto, CA). The cylindrical fiber power output was measured both before treatment and after completion of the illumination procedure using an integrating sphere power meter (Integra, Coherent Medical Group, Palo Alto, CA). The power density (mW/cm fiber) and light fluence (J/cm fiber) were calculated at the surface of the light diffusing fiber.

The local infusion of Lu-Tex was performed as described above. Twelve minutes post infusion, the optical fiber was introduced through the right carotid artery and advanced via fluoroscopic guidance to one of the iliac arteries. Light was delivered 15 min post LuTex infusion at 180 J/cm fiber at a rate of 200 mW/cm fiber. An additional animal received light only to one artery while another received a ‘sham’ treatment to one iliac artery. Two weeks following photoangioplasty treatment, the animals were euthanized, perfusion fixed with 10% buffered formalin and embedded in paraffin for histologic processing. Both iliac arteries, including a section of the lower abdominal aorta, were analyzed. The photoangioplasty site was confirmed by matching the anatomy with the respective fluoroscopic picture. Serial cross sections, taken every 0.5 cm, from the proximal end of the lower abdominal aorta to the bifurcation and then down each iliac artery were stained with hematoxylin and eosin (H&E). Immunohistochemical demonstration of macrophages was performed using the mouse monoclonal antibody RAM11 (Dako Corporation, Carpinteria, CA). RAM11 is a cytoplasmic antigen specific for rabbit macrophages [15]. Briefly, 4 μm formalin-fixed, paraffin-embedded serial sections were deparaffinized and incubated with primary RAM11 antibody (1:200) for 30 min. After incubation with biotinylated secondary anti-mouse IgG (Dako) for 10 min the sections were incubated with streptavidin horse radish peroxidase (Dako) for an additional 10 min. Following rinsing, the macrophages were stained with 3,3’-diaminobenzidine (Dako) and counterstained with filtered Mayer’s hematoxylin solution. Negative controls consisted of omission of the primary antibody. Slides were analyzed by light microscopy and planimetry using C-IMAGING system (Compix Inc. Imaging system, Mars, PA). Histomorphometric measurements were performed by skilled observers who had no knowledge of the treatment groups. Percent plaque area was defined as [(internal elastic lamina area—lumen area)/ (internal elastic lamina area)]×100. Each processed section was divided into quadrants for individual macrophage analysis. The percent area occupied by macrophages in both the intima and media were also quantified.

3. Results

3.1. Lu-Tex biodistribution

The levels of Lu-Tex in vessels and plasma, following local infusion (1.2 mg/kg) was measured using extraction and fluorescence analysis and are shown in Table 1. The
untreated contralateral iliac artery and lower abdominal aorta exhibited very little Lu-Tex uptake/retention compared to the vessel that received intra-arterial sensitizer treatment. Ratios of Lu-Tex in the treated iliac to the untreated contralateral iliac vessel were 36.6, 18.5 and 12.9 to 1 at immediate, 15 and 30 min post infusion, respectively. Sensitizer levels in the plasma may have resulted from overflow injection, equilibration with tissue or uptake into the vasa vasorum. The combined average Lu-Tex levels in treated arteries post infusion was significantly higher than that in the untreated iliac arteries (14.02±8.3 \( \mu \text{g/g} \) \( n=6 \), \( P=0.001 \)) and aorta (1.04±0.87 \( \mu \text{g/g} \) \( n=6 \), \( P=0.001 \)), but not compared to plasma (10.70±3.91 \( \mu \text{g/g} \) \( n=6 \), \( P=\text{ns} \)).

Fluorescence spectral bioimaging was used to evaluate the biolocalization of Lu-Tex in atherosclerotic lesions compared to the surrounding uninvolved tissue. Fig. 2a displays the black and white photo of the iliac luminal surface, including lower abdominal aorta, of a rabbit that was analyzed 15 min post Lu-Tex infusion. The area involved with atheroma is visualized and can be distinguished from the surrounding uninvolved vascular tissue. The fluorescence emission profiles from the atheroma and the adjacent uninvolved surface of the infused iliac artery was compared to the contralateral non-infused iliac artery (Fig. 2b). Little, if any, was detected in the untreated side. Quantitation of the fluorescence emission profiles (Fig. 2b) obtained from atheroma and uninvolved adjacent aortic wall revealed a greater than 40:1 ratio of Lu-Tex fluorescence in plaque compared to uninvolved vessel.

Table 1
Lu-Tex biodistribution after local delivery

<table>
<thead>
<tr>
<th>Group</th>
<th>Lu-Tex-injected Iliac (( \mu \text{g/g} ))</th>
<th>Contralateral Iliac (( \mu \text{g/g} ))</th>
<th>Aorta (( \mu \text{g/g} ))</th>
<th>Plasma (( \mu \text{g/ml} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no Lu-Tex)</td>
<td>0.04±0</td>
<td>0.065±0.04</td>
<td>0.03±1.5</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>Immediately</td>
<td>21.6±5.7</td>
<td>0.59±0.014</td>
<td>1.64±1.5</td>
<td>13.8±2.0</td>
</tr>
<tr>
<td>15 min post</td>
<td>14.1±8.8</td>
<td>0.76±0.06</td>
<td>1.1±0.14</td>
<td>12.0±3.2</td>
</tr>
<tr>
<td>30 min post</td>
<td>6.32±0.4</td>
<td>0.49±0.06</td>
<td>0.38±0.27</td>
<td>6.35±1.4</td>
</tr>
</tbody>
</table>

* Data represent the mean and S.D. of Lu-Tex levels in arterial segments; two rabbits were in each group. Lu-Tex (1.2 mg/kg) delivered in the iliac artery at an infusion rate of 0.2 ml/min.

Fig. 2. Fluorescence spectral bioimaging analysis of the iliac arteries 15 min following Lu-Tex (1.2 mg/kg) infusion in the left iliac artery. The arteries were exposed longitudinally (a), the fluorescence emission profiles of diseased and normal wall areas are shown in (b). Regions of plaque (red) and normal wall (pink) from the infused side are compared with regions of plaque (dark blue) and normal wall (light blue) from the contralateral side. A region from the lower abdominal aorta (green) is also shown.
Selective uptake of Lu-Tex within plaque was seen at all time points studied. In Fig. 3, the temporal biodistribution of Lu-Tex is shown. Selective accumulation/retention of LuTex was observed in atheroma compared to the adjacent wall tissue with some ‘washout’ from the infusion area.

3.2. Photoangioplasty

In each animal, one iliac artery was treated and results compared to the untreated contralateral artery and also additional light alone and ‘sham’ treated arteries. Photoangioplasty (180 J/cm^2 fiber and 250 mW/cm fiber) was performed in seven rabbits, 15 min following local infusion of the photosensitizer. One rabbit received light alone while another received a ‘sham’ treatment. Two weeks after photoangioplasty, histological analysis was performed. Although there was no difference in external elastic lamina area both in the treated lesions and non-treated contralateral lesions (3.4±1.0 (n=40) versus 3.7±0.9 mm^2 (n=32), P=ns), the mean percent plaque area in the treated segments was significantly less than in the nontreated contralateral segments (73±10% (n=40) versus 82±12% (n=36), P<0.001). The mean percent plaque area in the light alone segments was 79±3% (n=12) while in the no treatment arteries it was 82.4±5% (n=18). The reduction in plaque is small, but statistically significant. The treatment effect in the seven rabbits was analyzed using a two-tailed paired t-test. A statistical significant decrease in mean plaque area in the treated arteries compared to the contralateral control arteries was found (P=0.02). No damage to the medial wall was detected following histologic inspection of the H&E sections. The plaque area in the lower abdominal aorta was 62.5% (n=10). Fig. 4 shows results of specific macrophage staining using an immunochemical marker (RAM11). The depletion, if any, of other cell types is currently being investigated. Quantitative analysis revealed significant reduction of macrophages in the treated lesion compared to the nontreated contralateral lesion in both the intima (5.1±5.3% (n=113) versus 22±15% (n=139), P<0.01) and media (7.7±10% (n=113) versus 23±19% (n=139), P<0.01). Analysis using a two-tailed paired t-test also revealed a statistical significant decrease in macrophage area in both the intima and medial of the treated arteries compared to the contralateral control arteries (P<0.05 for both groups). All the treated arteries had statistically significant macrophage reduction compared to the light alone and ‘sham’ arteries.
4. Discussion

The present study was undertaken to assess the efficacy and temporal biodistribution of locally delivered Lu-Tex and subsequent photoangioplasty in a rabbit balloon injury model of atherosclerosis. Lu-Tex selectively localized in atheroma compared to adjacent normal vessel within the infused iliac artery. The contralateral, uninfused iliac arteries showed minimal uptake in both diseased and normal portions of the vessel. These data indicate that local arterial infusions can selectively deliver sensitizer to the diseased area where uptake is limited to atheromatous plaque. Selectivity for plaque occurred despite high local concentrations in the infused iliac artery with minimal uptake in normal adjacent vessel. Subsequent photoactivation of Lu-Tex at the diseased artery caused a small, but statistically significant, reduction in plaque burden as assessed by histology. Moreover, a dramatic reduction in macrophages in both the intima and media was seen in the photoangioplasty treated lesions as compared to non-illuminated arteries. Local delivery has the advantage of reducing the dose and decreasing the chance of any potential systemic toxicity. One-tenth of the systemic dose was chosen arbitrarily and further reduction of dose may be feasible.

The actual mechanism of Lu-Tex uptake and retention in atherosclerotic lesions has not as yet been fully defined. However, Lu-Tex plasma lipoprotein binding, to both LDL and HDL, or modified and aggregated lipoproteins may play important roles. Rabbits fed a hypercholesterolemic diet have more Lu-Tex associated with the LDL fraction than those animals fed normal dietary chow (40.5% compared to 16.9%) [8]. In contrast, less Lu-Tex was bound to the HDL (5.3%) in atherosclerotic animals than in normal animals (24.5%). Hypercholesterolemic animals down-regulate the genes for LDL receptors; however, macrophages, foam cells and smooth muscle cells possess cell-surface molecules called scavenger receptors, the expression of which is not regulated by cholesterol levels [16]. Oxidized LDL is recognized by macrophage scavenger receptors and recently it has been suggested that certain scavenger-type receptors recognize and engulf some of the inner cholesterol ester component of HDL, a phenomenon known as selective lipid uptake [16]. In addition, aggregated and oxidized LDL may be accumulated by macrophages by a phagocytic process. Further studies are needed to evaluate the role of lipoproteins in plaque retention following local delivery of the photosensitizer. The actual cell death mechanism, induced by photosensitization, may also be related to the presence and generation of oxidized lipids. Cells are extremely sensitive to oxidized lipids and their presence within the cell can induce apoptosis [17]. Photoangioplasty initiates many lipid peroxidation reactions. It has previously been reported that photodynamic therapy with Lu-Tex induced apoptosis in sarcoma-bearing mice [18]. Lu-Tex photoangioplasty of atheromatous plaque may further stress an already heavily oxidatively-stressed environment, causing apoptosis, although this has yet to be fully confirmed.

Many cardiovascular events are due to the rupture of unstable soft plaque [19]. The role of macrophages in unstable plaque syndrome is presently being realized as being extremely important. Richardson et al. [20] reported macrophages at the site of plaque rupture in atherosclerotic lesions from 85 postmortem coronary specimens. Van der Wal et al. [21], after studying the vessels of 20 patients who died from acute myocardial infarction, reported that macrophages were the predominant cells at the immediate site of either rupture or superficial erosion of a fibrous cap. Moreno et al. [22] studied atherectomy specimens from culprit lesions responsible for unstable angina, non-Q wave myocardial infarction, and stable angina and demonstrated that macrophages are increased in coronary plaque from patients with unstable syndrome compared with plaque from patients with stable angina.

Several studies have also demonstrated the role of macrophages in early stage neointimal hyperplasia after angioplasty and stent implantation. Rubin et al. [23] reported that 24 h after angioplasty, there is an apparent migration of macrophages in the adventitia, and after 1 week, the macrophages migrated in to all three layers of the vascular wall, especially into the intima in rats. Kollum et al. [24] also reported the role of macrophages and apoptosis in rabbits. They concluded that macrophage accumulation and apoptosis in the early phase after stent implantation in rabbits appears to play a role in extracellular matrix secretion, which increases the neointima formation after 4 and 12 weeks compared with balloon angioplasty.

Clinical studies also support the critical role of the macrophage in restenosis following intervention [4,25]. Moreno et al. [25] reported that macrophage content was greater in unstable angina than in stable angina. They also demonstrated that macrophages are increased in coronary atherectomy tissue from vulnerable lesions that develop restenosis, and that macrophage-rich areas were larger in plaque from patients with restenosis than in plaque from patients without restenosis [25].

Lu-Tex photoangioplasty has been shown to cause plaque resolution in both hyperlipidemic and post-balloon injury models. Currently, Lu-Tex photoangioplasty is being studied in clinical trials for patients with peripheral [26] and coronary arterial disease. In contrast to mechanical techniques, Lu-Tex photoangioplasty appears to reduce plaque,atraumatically, without damage to the vessel wall and endothelium. Moreover, the mechanism of action appears to be associated with elimination of macrophages from the lesions.

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